THE UNIVERSITY OF ALBERTA

STUDIES ON THE RELATIONSHIP BETWEEN PORPHYRIN BIOSYNTHESIS AND ACUTE PORPHYRIA

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA

MAY, 1965

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Studies on the Relationship between Porphyrin Biosynthesis and Acute Porphyria" submitted by Edward George Hunter in partial fulfillment of the requirements for the degree of Master of Science.

ABSTRACT

Acute intermittent porphyria is a disease inherited as a Mendelian dominant trait and it is characterised by abdominal and neurological symptoms and by the excretion of the porphyrin precursors, δ-aminolevulinic acid and porphobilinogen. However neither δ-aminolevulinic acid nor porphobilinogen appear to be responsible for the symptoms observed in this disease and the purpose of part I of this thesis was to examine the possibility that products derived from these porphyrin precursors might be responsible for some of the symptoms of acute porphyria.

γ, δ-Dioxovaleric acid is derived from the in vivo transamination of δ-aminolevulinic acid and in view of a report that it can precipitate an attack of porphyria it has been synthesised. Under non-enzymic conditions two molecules of δ-aminolevulinic acid condense to a dihydropyrazine which autoxidises to a pyrazine. A method has been developed for the synthesis of the pyrazine and the dihydropyrazine has been synthesised by the method of Scott. Under non-enzymic conditions porphobilinogen condenses to porphobilin, a substance which has previously been reported to be pharmacologically active. However since it was originally isolated from urine it was considered likely that the activity was due to histamine contamination. We have prepared porphobilin from a sample of synthetic porphobilinogen according to a modification of the method of Waldenstrom et al.[28] These four compounds viz., γ, δ-dioxovaleric acid, dihydropyrazine, pyrazine and porphobilin were tested for their effects on the blood pressure of an anaesthetised cat and for their effects on isolated intestinal strips. However none of these compounds were active. The inactivity of porphobilin derived by a synthetic method provides evidence that the activity of porphobilin, isolated from urine, was due to contamination with a pharmacologically active substance.

The over-production of porphyrins and porphyrin precursors in the livers of animals, fed porphyria-inducing drugs, results from an enhanced synthesis of the first enzyme of the porphyrin

biosynthetic chain. These drugs appear to interfere with the repressor control mechanism for the synthesis of this enzyme. The structure activity-relationships of a series of structural analogues of the porphyria-inducing compound 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine have previously been studied in our laboratory. These analogues differed from one another by the size of the 4 substituent.

In part II of this thesis a study of Fisher-Taylor-Hirschfelder models and ultraviolet spectra of these compounds indicated that substitution at the 4 position caused a twisting of the 3 and 5 ethoxycarbonyl substituents out of the plane of the dihydropyridine ring. The possibility that non-planarity was responsible for porphyria-inducing activity was tested by 1) increasing the size of the 4 substituent, 2) increasing the size of the 2 and 6 substituents without alteration of the 4 substituent. The porphyria-inducing activity of these compounds was tested by two methods: 1) by feeding these compounds to guinea-pigs and estimating the urinary excretion of δ-aminolevulinic acid and porphobilinogen, 2) by measuring the porphyrin accumulation in cultures of chick embryo liver cells treated with the compounds. The results of these tests indicated that the non-planar relationship is necessary for optimal porphyria-inducing activity.

ACKNOWLEDGEMENTS

The author wishes to express appreciation and gratitude to G. S. Marks, D. Phil., for his kindly counsel, his constant encouragement and, above all, his unfailing patience, all of which have contributed so much to the completion of this thesis. A sincere thanks is also tendered to D. P. MacLeod, Ph. D., who has co-operated so generously in planning the pharmacological evaluation of the compounds employed in this work.

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INTRODUCTION

Acute intermittent porphyria is a disease inherited as a Mendelian dominant trait. It is characterised by abdominal and neurological symptoms as well as by an abnormal porphyrin metabolism and patchy demyelination of the nervous system.

This disease has aroused the curiosity and interest of clinicians and a great deal of work has been done to identify the metabolites excreted. While early work was hampered by a lack of knowledge of normal porphyrin metabolism the recent clarification of the steps in the biosynthesis of heme have facilitated studies in this field. The elucidation of the early steps in the porphyrin pathway resulted from the isotope tracer studies of Shemin and Wittenberg.[1] These studies showed that the four nitrogen atoms of protoporphyrin were derived from the nitrogen atoms of four molecules of glycine (1) and that the carbon atoms of heme (3) were derived from the α-carbon atoms of glycine and from the carbon atoms of succinic acid (2). The carboxyl carbon atom of glycine was not utilised in the biosynthesis. By a complete degradative procedure Shemin and Wittenberg[2] were able to locate the carbon atoms of each of the above precursors in the heme molecule. The origin of each of the atoms of heme as disclosed by these studies is indicated in figure 1.

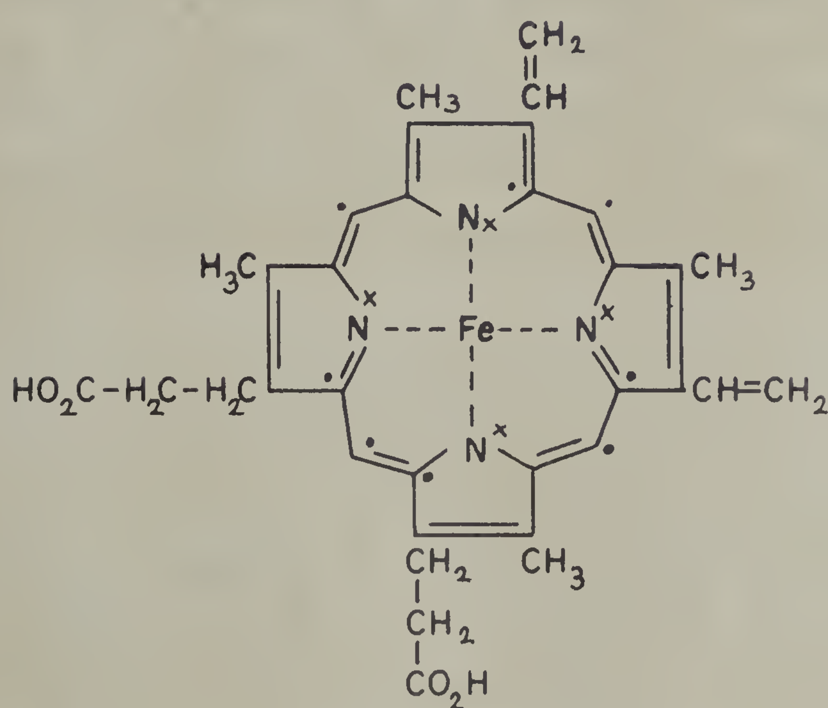
From the labelling pattern in heme it was deduced that δ-aminolevulinic acid (4) could be a precursor of heme[3] and this deduction was confirmed in 1955 by the observation that δ-aminolevulinic acid could replace succinic acid and glycine for porphyrin biosynthesis.[4]

$$H_2NCH_2COCH_2CH_2CO_2H \qquad (4)$$

It was subsequently shown[5] that the first step of porphyrin biosynthesis involves the condensation of glycine and succinyl CoA (5) to δ-aminolevulinic acid by the enzyme δ-aminolevulinic acid synthetase. δ-Aminolevulinic acid

$$\overset{*}{N}H_2 \cdot \dot{C}H_2 \cdot CO_2H \qquad (1)$$

$$HO_2C \cdot CH_2 \cdot CH_2 \cdot CO_2H \qquad (2)$$



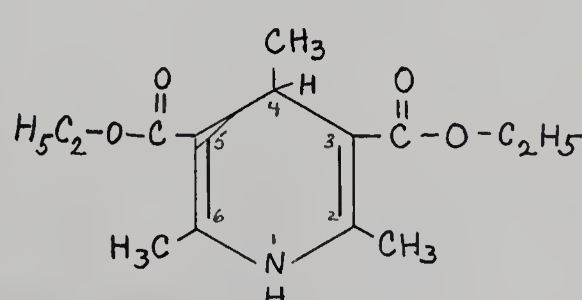
(3)

Figure I :

Origin of carbon and nitrogen atoms of heme.
Labelled carbon and nitrogen atoms derived from the nitrogen atom and the α c-atom of glycine.
Unlabelled carbon atoms derived from succinic acid.
(after D. Shemin[17])

dehydrase catalyses the condensation of two molecules of δ-aminolevulinic acid to yield the monopyrrole porphobilinogen (7). Under the influence of two enzymes[6], uroporphyrinogen synthetase and uroporphyrinogen isomerase, four molecules of porphobilinogen are condensed to form the macrocycle, uroporphyrinogen III (8). Uroporphyrinogen decarboxylase[7] is responsible for the production of coproporphyrinogen III (9) by decarboxylation of the acetic acid residues of uroporphyrinogen III. Oxidation, by the enzyme coproporphyrinogen oxidase, of the two propionic acid groups in rings A and B of Coproporphyrinogen III to vinyl groups and of the methylene bridges to methine bridges, affords protoporphyrin IX (10). The final step in the biosynthesis of heme is the enzymic incorporation of ferrous iron into protoporphyrin IX. The various steps in this biosynthetic pathway are depicted in figure 2.

With the steps of porphyrin biosynthesis clarified it was possible for Granick and Urata[8,9] to study the metabolic defect occurring in acute porphyria. They were able to produce a disorder of porphyrin metabolism in the livers of guinea pigs, resembling that seen in acute intermittent porphyria, by the administration of the porphyrinogenic compound 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC, 12).



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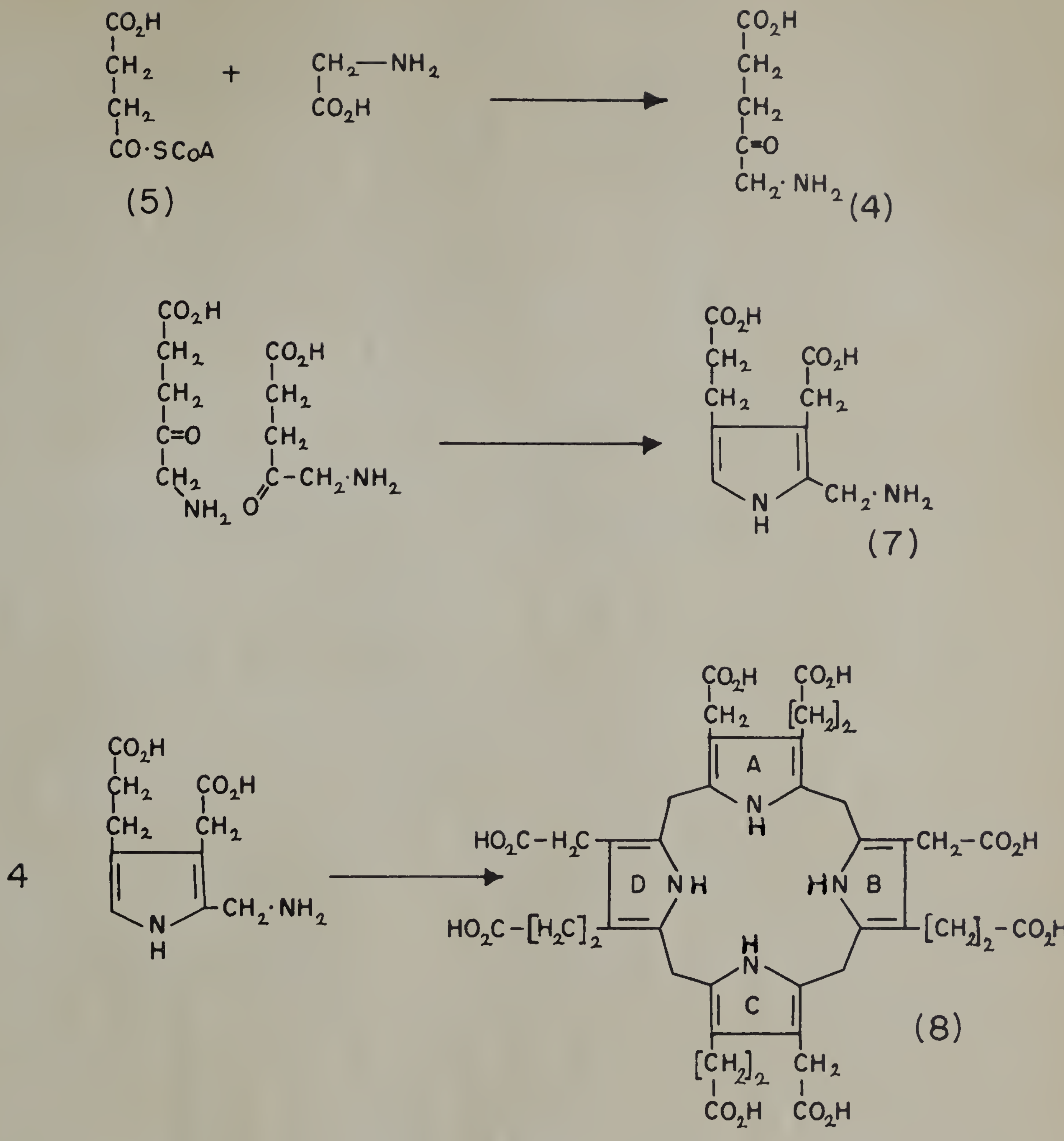


Figure 2. Biosynthesis of Heme

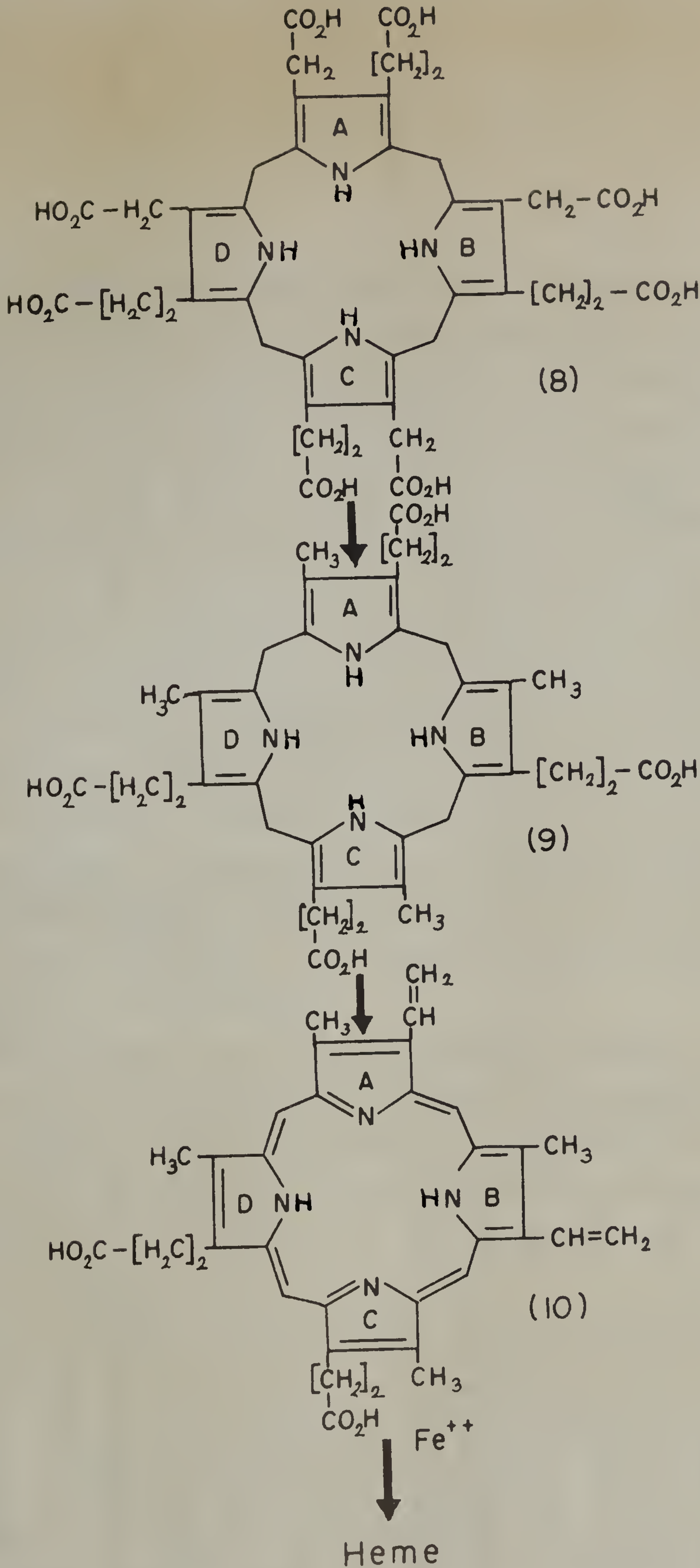


Figure 2: Biosynthesis of Heme, cont'd

The level of the liver enzymes involved in porphyrin biosynthesis was found to be normal with the exception of the first enzyme of the pathway viz., δ-aminolevulinic acid synthetase. Whereas in normal guinea pig liver this enzyme could barely be detected its level in the porphyric guinea pig was elevated forty-fold. It thus appears that porphyrin biosynthesis is normally controlled by the level of δ-aminolevulinic acid synthetase and that the overproduction of porphyrins and porphyrin precursors in porphyria is the result of an increased activity of this enzyme. This increased activity of δ-aminolevulinic acid synthetase induced by DDC could have resulted from one of several factors: (a) DDC might have activated a normally inactive form of δ-aminolevulinic acid synthetase. (b) DDC may have interfered with a hormonal mechanism which normally controls the activity of δ-aminolevulinic acid synthetase. (c) DDC may have stimulated a *de novo* synthesis of the enzyme.

To investigate the first possibility normal liver mitochondria[8], in which the enzyme is located, were incubated in the presence of DDC. Since no increase in the activity of δ-aminolevulinic acid synthetase was observed it was apparent that the drug did not increase the activity of the enzyme by the activation of an inactive enzyme.

Tissue culture of chick liver parenchyma cells in the presence of DDC resulted in an increase in activity of δ-aminolevulinic acid synthetase and consequently in the production of large amounts of porphyrins. This experiment showed that the action of this chemical is a direct one on the liver cells rather than an indirect one mediated through influencing hormone action.

To investigate the possibility that DDC induced a *de novo* synthesis of δ-aminolevulinic acid synthetase, chick liver parenchyma cells were cultured in the presence of

Mitomycin C, Actinomycin D and other inhibitors of protein synthesis. Addition of DDC to these cells did not cause any increase in the activity of δ-aminolevulinic acid synthetase. It was thus apparent that the mode of action of this chemical on normal cells capable of carrying out protein synthesis was by direct stimulation of the synthesis of δ-aminolevulinic acid synthetase.

On the basis of these experiments with drug-induced porphyrias Granick[10] suggests that there is a mechanism for the control of this enzyme by repression of its formation and that these drugs interfere with the repressor control mechanism.

He has further suggested that in the hereditary disease of acute intermittent porphyria the regulatory control mechanism governing the activity of the structural gene for δ-aminolevulinic acid synthetase is defective and does not sufficiently repress the formation of this enzyme.

A crucial question which remains unanswered in acute intermittent porphyria concerns the nature of the relationship between the metabolic derangement and the symptoms observed.

Early workers believed that the clinical symptoms resulted from the accumulation of porphyrins or porphyrin precursors in the body. This view has been challenged and alternative hypotheses to explain this relationship have been advanced by Goldberg[11], and by Rimington and De Matteis.[12] These theories are presented below.

1. The Hypothesis of Goldberg.[11]

Goldberg suggests that porphobilinogen is a precursor of a nutrient ("Substance X") of myelin and that the enzymic conversion of porphobilinogen to Substance X is prevented in patients with acute porphyria. This defect is considered to be responsible for the accumulation and subsequent excretion

of porphobilinogen and for demyelination. Goldberg cites evidence showing that the clinical symptoms observed in acute intermittent porphyria may be explained on the basis of demyelination. Thus the demyelination of peripheral nerves, demonstrated at post mortem or by biopsy may explain the limb paralysis. Lesions observed in the phrenic nerve could be responsible for respiratory paralysis which is the cause of death in cases where the disease is fatal. Demyelination of preganglionic motor fibres which innervate the viscera may be responsible for the gastrointestinal symptoms observed.

2. The Hypothesis of Rimington and De Matteis.[12]

These workers suggest that both the clinical symptoms and the metabolic derangement are a result of a defect in the production, availability or utilization of acetyl-CoA. Since acetyl-CoA is a precursor of acetylcholine a critical reduction in acetylcholine synthesis is thought to occur leading to the clinical symptoms. The metabolic derangement is explained as follows: condensation of glycine with succinyl-CoA to δ-aminolevulinic acid is an example of a general reaction of glycine, e.g. glycine also condenses with acetyl-CoA to give aminoacetone, an analogue of δ-aminolevulinic acid (figure 3).

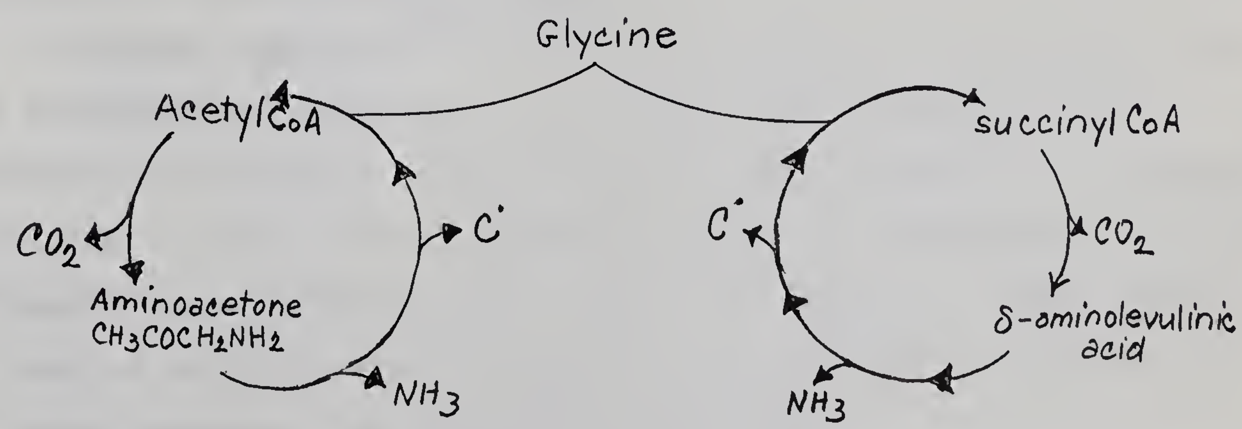


Fig. 3 Hypothesis of Rimington and DeMatteis
C' = α Carbon atom of glycine
(to purines, formate etc.)

It is suggested that since there is a reduced availability of acetyl-CoA the amounts of glycine utilized for aminoacetone synthesis will be decreased and more will be available for reaction with succinyl-CoA to form δ-aminolevulinic acid and hence porphyrins.

In view of the recent demonstration by Granick and Urata that the accumulation of porphyrins and porphyrin precursors is the result of an over-production of the enzyme δ-aminolevulinic acid synthetase, the theories of Goldberg, and Rimmington and De Matteis require revision. Moreover, the demonstration that the accumulation of porphyrins and porphyrin precursors in acute intermittent porphyria is the result of an over-production rather than an under-utilization of these substances leads one to reconsider the early hypothesis that the clinical manifestations of this disease might be directly caused by some porphyrin metabolite.

During an attack of porphyria, patients usually excrete large quantities of porphobilinogen and δ-aminolevulinic acid. The excretion of these substances is usually in direct proportion to the severity of the symptoms, suggesting a causal relation (see Table 1).

However neither porphobilinogen[13] nor δ-aminolevulinic acid[37] have any activity on isolated organs or on the blood pressure of the anaesthetised cat.

Several authors[14,15,16] have claimed that porphyrins have pharmacological activity since they influence the intestine or uterus. These results are thought by Goldberg[13] to be due to the use of impure porphyrins, probably containing histamine as a contaminant, since he failed to demonstrate any action of purified porphyrins on isolated organs or on the blood pressure of anaesthetised cats.

Goldberg _et al._[13] have also considered the possibility that an as yet unidentified metabolite might be responsible for the clinical manifestations. To test this possibility

Sex	Age	Clinical Status	Urine PBG* qualitative	PBG mg/l	ALA† mg/l
M	28	Acute attack	+++	194	219
F	47	Acute attack	+++	99	45
M	29	Acute attack	++	40	41
F	35	Acute attack	++	138	81
M	29	Acute attack	++	31	30
F	43	Mild attack	+	25	26
F	42	Recent attack	+	20	8
F	35	Recent attack	Neg.	6	7
M	29	Recent attack	Trace	24	81
M	28	Recent attack	Neg.	2	7
M	52	Mild pain	Neg.	3	3
F	39	Mild pain	Neg.	8	5
M	23	Mild pain	Neg.	6	11
F	35	Latent	Neg.	2	2
M	54	Latent	Neg.	1	5
F		Latent	Neg.	0	1
M	29	Latent	Neg.	2	3
F	33	Latent	Neg.	1	1
M	49	Latent	Neg.	1	2

Table 1

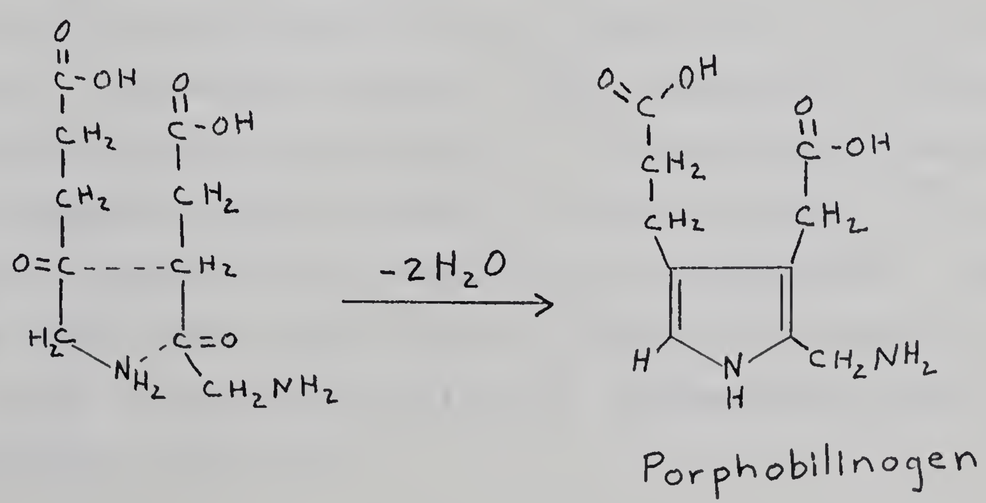
Urinary Excretion of δ-aminolevulinic Acid and Porphobilinogen in the South African Type of Porphyria (R. Schmid [44])

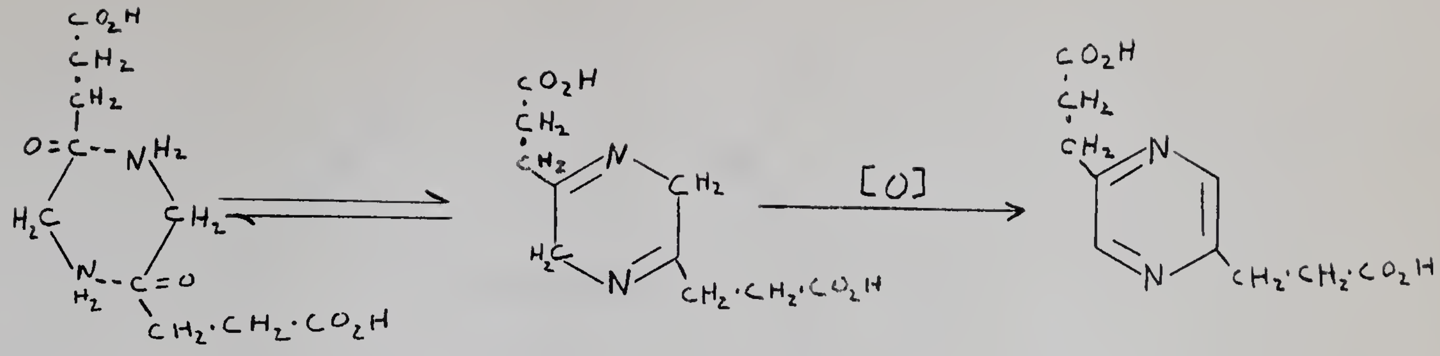
*PBG = porphobilinogen

†ALA = δ-aminolevulinic acid

a sterile sample of urine from a patient suffering an acute attack of porphyria was infused into an anaesthetised cat; no change in blood pressure was observed. On the basis of this observation these workers conclude that they have ruled out the possibility of an unknown metabolite being responsible for the symptoms observed. The conclusions of Goldberg et al.[13] appear to be unjustified since any unknown pharmacologically active substances present in the urine might have been destroyed in the sterilization process.

The objective of Part I of this thesis was to reinvestigate the possibility that some porphyrin metabolite or a derivative of such a metabolite was responsible for the clinical manifestations of porphyria. Since the primary metabolic lesion involves the over-production of δ-aminolevulinic acid synthetase and consequently of δ-aminolevulinic acid we were interested in investigating the possibility that some compound derived from δ-aminolevulinic acid might be responsible for the symptoms observed. It has been pointed out above that two molecules of δ-aminolevulinic acid are condensed enzymically to yield porphobilinogen. However under non-enzymic conditions two molecules of δ-aminolevulinic acid condense to a dihydropyrazine (13) which autoxidises to a pyrazine (14).





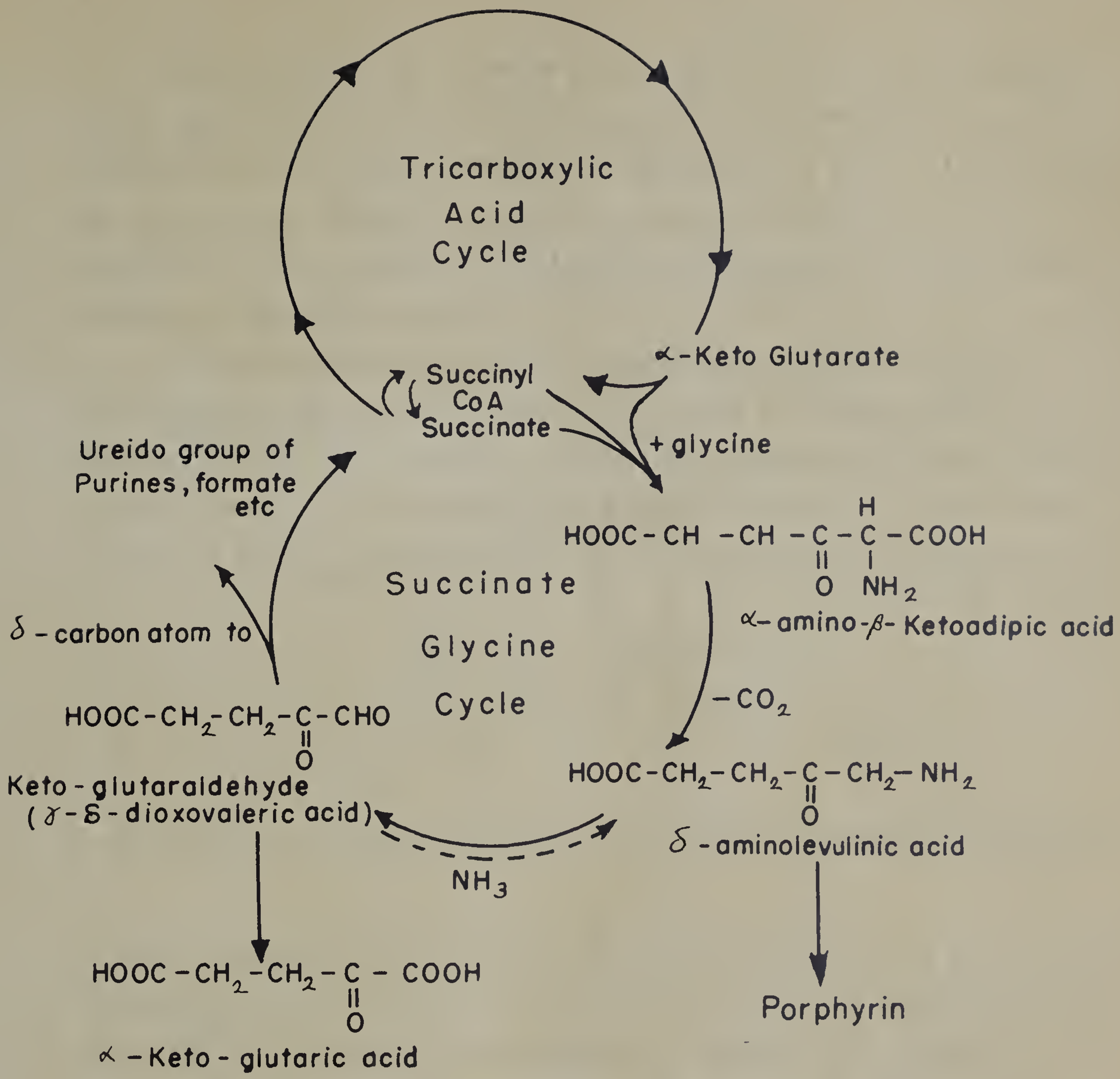
2 δ-Aminolevulinic acid   Dihydropyrazine (13)   Pyrazine (14)

Condensation of two δ-aminolevulinic acid molecules enzymically to porphobilinogen or non-enzymically to a dihydropyrazine which autoxidizes to a pyrazine (ref. 34).

The irreversible oxidation of a dihydropyrazine to a pyrazine would be facilitated by the apparent breakdown in cellular antioxidant systems in porphyria which results in the oxidation of porphyrinogens to porphyrins. The dihydropyrazine and pyrazine compounds derived from δ-aminolevulinic acid have been synthesised and tested for pharmacological activity and these experiments are reported in Part I of this thesis.

In an attempt to unify the reactions of glycine, Shemin[17] postulated a series of reactions, called the succinate-glycine cycle (figure 4). According to this postulate, δ-aminolevulinic acid, in addition to its utilisation for porphyrin synthesis, can be further metabolised in such a manner that its δ-carbon atom (originally from the α-carbon atom of glycine) is utilised for the synthesis of the ureido-group of purines, the β-carbon atom of serine, and methyl groups; the remaining 4-carbon residue is reconverted into succinate. Although certain experimental results support the existence of this cycle[17,18], its general metabolic significance remains to be evaluated. Enzyme studies have been restricted to the first reaction of this cycle, that is the conversion of δ-aminolevulinic into γ,δ-dioxovaleric acid (15).

$$OHC\text{-}\overset{\overset{O}{\|}}{C}\text{-}CH_2\text{-}CH_2\text{-}CO_2H \qquad (15)$$



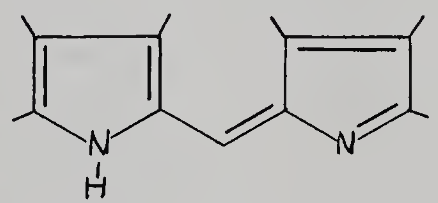
Succinate - Glycine Cycle; a pathway for the metabolism of glycine

(after D. Shemin ref. 17)

Fig 4

Richards[19] has demonstrated that γ, δ-dioxovaleric acid, when fed to a porphyric patient in remission could precipitate an acute attack. Consequently γ, δ-dioxovaleric acid has been prepared and its pharmacological activity evaluated. The results of these experiments are recorded in Part I of this thesis.

As mentioned above four moles of porphobilinogen condense enzymically to yield one mole of uroporphyrin III. However under non-enzymic conditions porphobilinogen forms porphobilin, a violet-black pigment, thought on the basis of its chemical properties[20,21] to be a dipyrrylmethene. (16)



(16)

Diagramatic representation of a dipyrrylmethene structure.

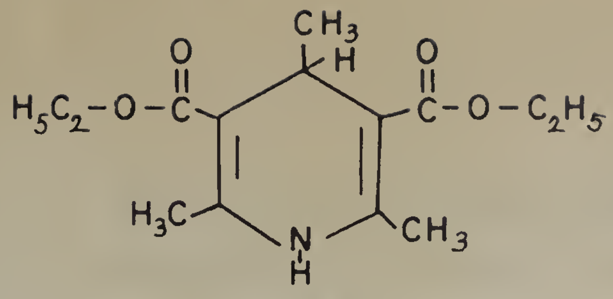
Goldberg *et al.*[13] investigated the possibility that porphobilin might be responsible for some of the clinical manifestations of acute intermittent porphyria. Porphobilin produced a contraction of guinea-pig ileum but since the contraction was blocked by the anti-histamine, Mepyramine, Goldberg considered the activity of the porphobilin (isolated from porphyric urine) to be due to contamination with histamine. To re-investigate the possibility that porphobilin possesses pharmacological activity, histamine-free porphobilin was prepared from a sample of synthetic porphobilinogen* and

*Synthesized and kindly supplied by S. F. MacDonald[22], N.R.C., Ottawa.

was tested according to the procedure of Goldberg et al. The results of these experiments which are not complicated by the possibility of histamine contamination are described in Part I of this thesis.

As mentioned above, the overproduction of porphyrins and porphyrin precursors in the livers of animals, fed porphyria-inducing drugs, resulted from an enhanced synthesis of δ -aminolevulinic acid synthetase; it was suggested that these drugs interfere with a repressor mechanism for the control of this enzyme. An important question which remains to be answered concerns the nature of the cellular receptor with which the porphyria-inducing drugs interact to interfere with the repressor control mechanism. Modern attempts to visualise a drug-receptor in molecular terms are based on the assumption that small molecules whose biological action is specific and structure-dependent display a high degree of molecular complementarity towards the site at which they act. A study of the structure-activity relationship of porphyria-inducing drugs is therefore a means with which the chemical nature of the receptor may be explored.

This consideration has led to further studies[23] of the structure-activity relationship in porphyria-inducing drugs, and since 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC, 17) is the most active drug it was chosen as the starting point for the investigation. The first analogue investigated[23] 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (18) differed from the parent dihydropyridine (17) by the replacement of the 4-methyl substituent with a hydrogen atom. This modification in structure resulted in a considerable loss of activity, and a similar effect was noted in the corresponding pyridine compounds (19,20). However replacement of the 4-methyl



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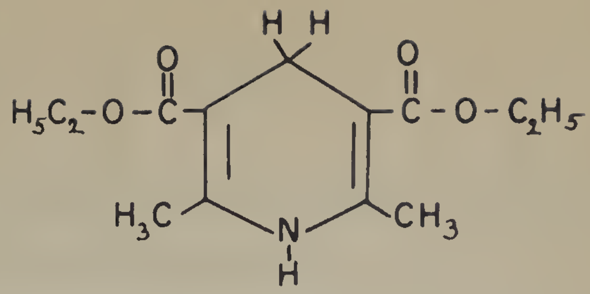

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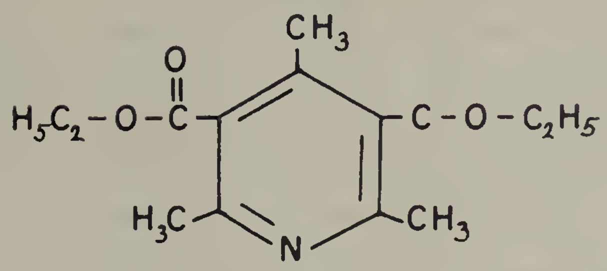

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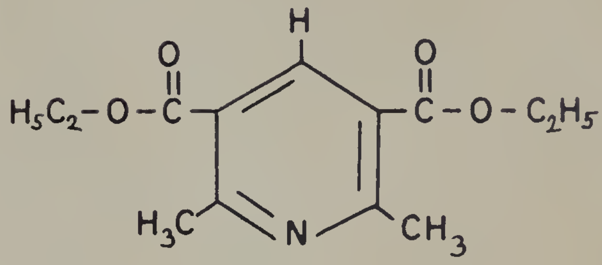

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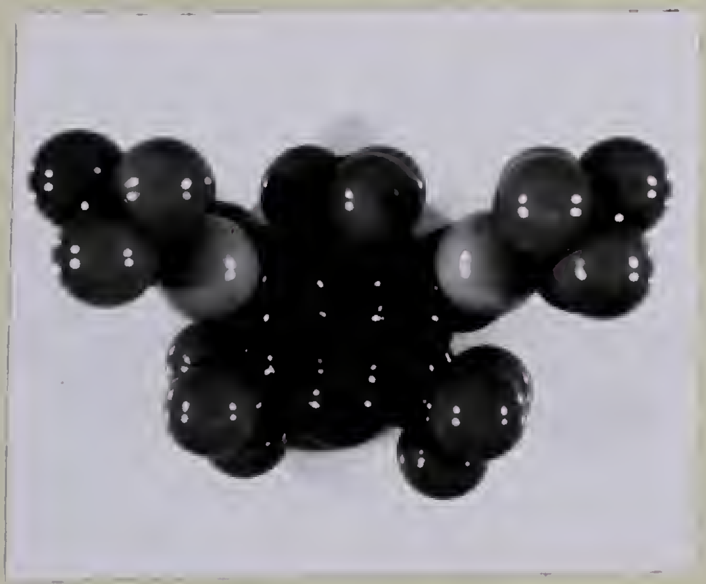
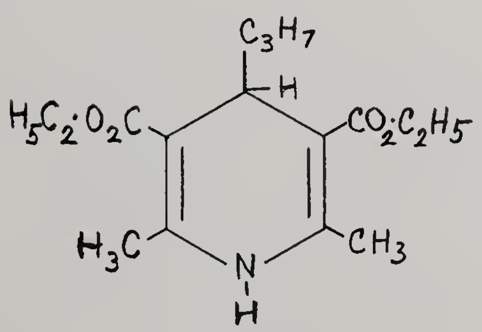


3,5 - diethoxycarbonyl
-2,4,6- trimethyl - pyridine
(19a)

3,5 - diethoxycarbonyl
-2,6 - dimethyl - pyridine
(20a)

substituent of dihydropyridine (17) by a 4-ethyl substituent[23] resulted in a marked increase in activity.

In the present investigation Fisher-Taylor-Hirschfelder models of several of these analogues (19a, 20a) were examined in an attempt to explain the variation in the activity. This examination revealed that because of the presence of the 2 and 6-methyl substituents, the introduction of a 4-alkyl substituent causes a twisting of the ethoxycarbonyl substituents out of the plane of the ring. This indicated that for optimal porphyria-inducing activity a nonplanar relationship between the ethoxycarbonyl substituents and the pyridine or dihydropyridine ring was essential. It was thus likely that if this interpretation was correct then the 2,4 and 6-methyl substituents of DDC could be replaced by substituents of equal or larger size with retention of activity. To test this idea 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-propylpyridine (21), 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-phenylpyridine (22) and 3,5-diethoxycarbonyl-1,4-dihydro-2,6-diethyl-4-methylpyridine (23) were synthesized and their porphyria-inducing activity evaluated. The results of this study are reported in Part II of this thesis.



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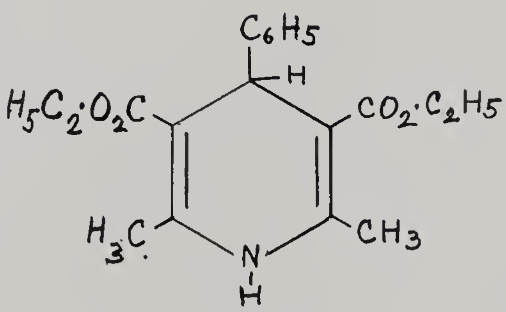

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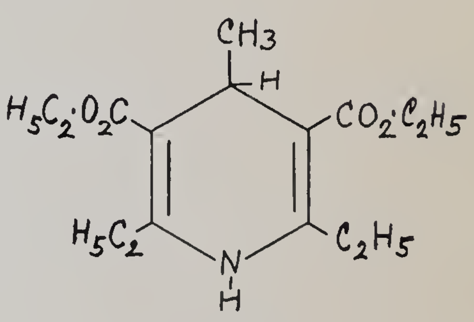


(23)

Furthermore a study of the ultraviolet spectra of these analogues was carried out which provided additional evidence for the nonplanarity of the ethoxycarbonyl substituents in the active porphyria-inducing compounds. This study is reported in Part II of this thesis.

MAIN SECTION

Part I

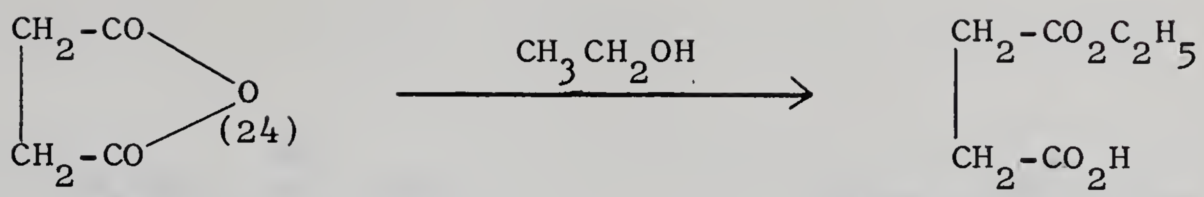
(a) THE SYNTHESIS OF δ-AMINOLEVULINIC ACID

(according to the procedure of D. Shemin[4,36])

The starting point for the synthesis of δ-aminolevulinic acid was succinic anhydride (24), which was converted into ethyl hydrogen succinate (25). Treatment of the latter with thionyl chloride gave β-carbethoxypropionyl chloride (26) which on condensation with ethylmagnesiomalonate (27) afforded ethyl β-keto-α carbethoxy-adipate (28). This was converted into β-keto adipic acid (29) by treatment with concentrated hydrochloric acid. Treatment of the latter product (29) with ethyl nitrite yielded δ-oximinolevulinic acid (30) which after reduction with stannous chloride and hydrochloric acid gave δ-aminolevulinic acid hydrochloride (31)

(b) THE SYNTHESIS OF PYRAZINE-3,6-DIPROPIONIC ACID

A method that has previously been used for the synthesis of pyrazines is that of Dakin and West.[24] The method consists in treating an aminoketone with 4N-ammonium hydroxide overnight in the presence of air, whereupon condensation to the pyrazine takes place. A typical example, 2,5-dibenzyl-3,6-dimethylpyrazine (32) has been prepared by the condensation of two molecules of α-benzyl-α-aminoacetone (33).



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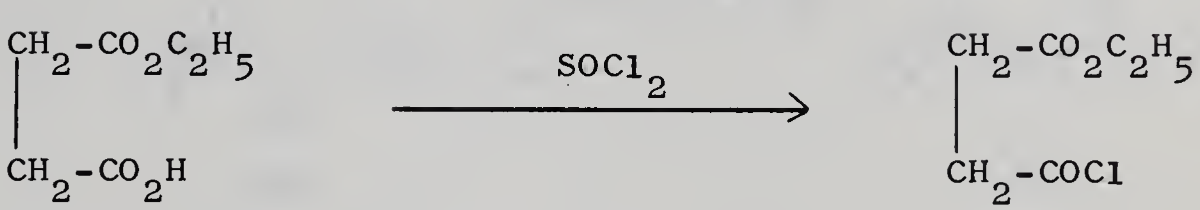

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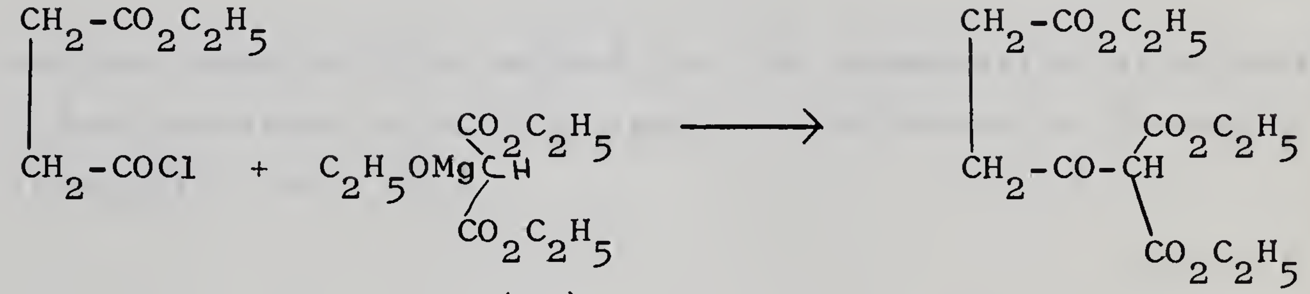

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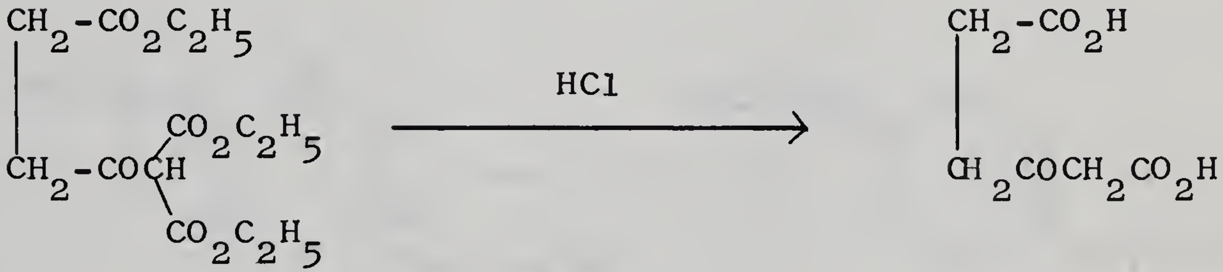

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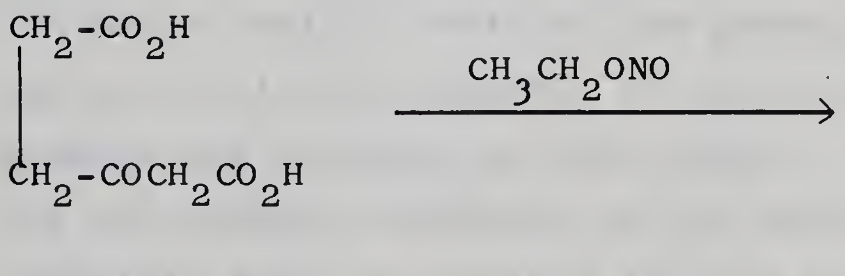

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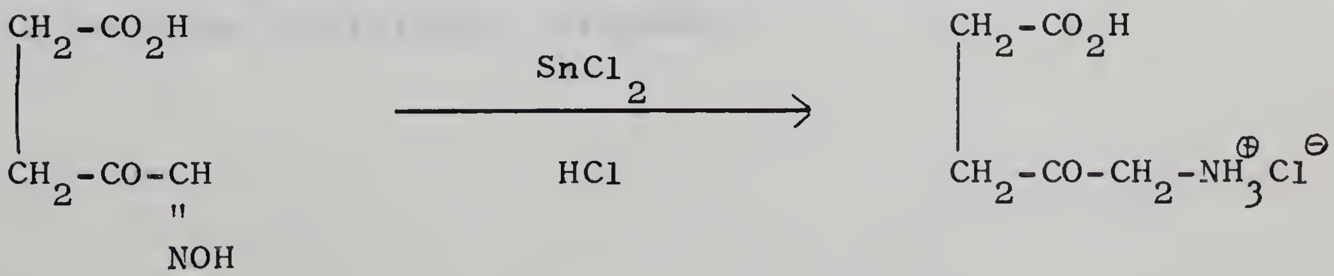

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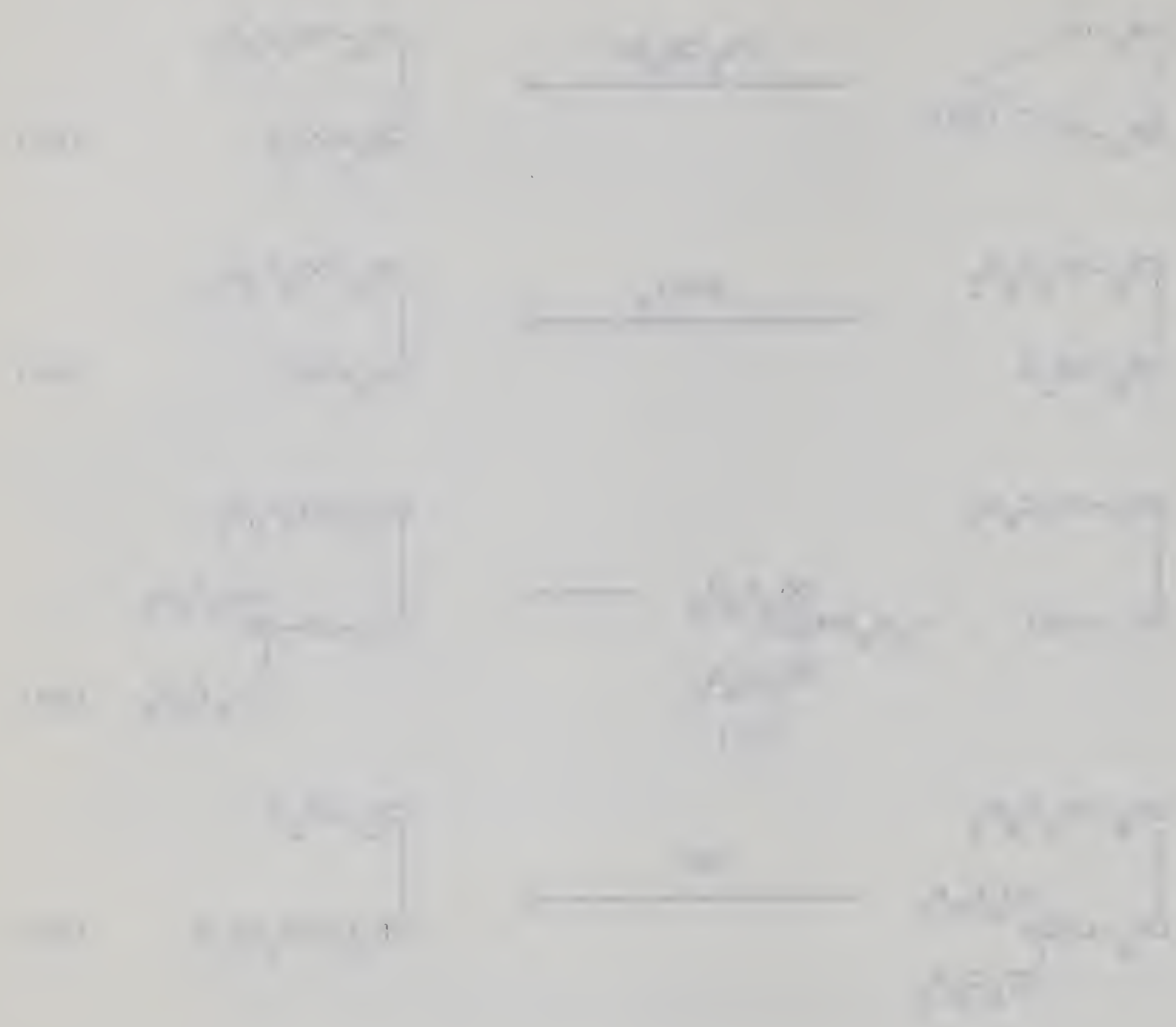
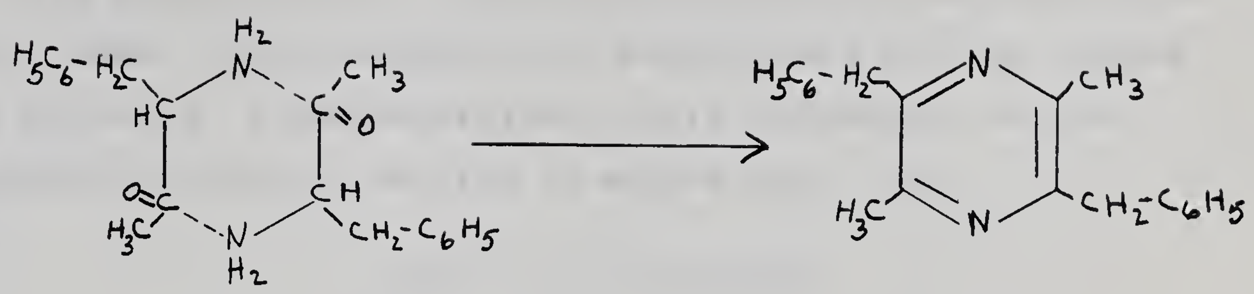
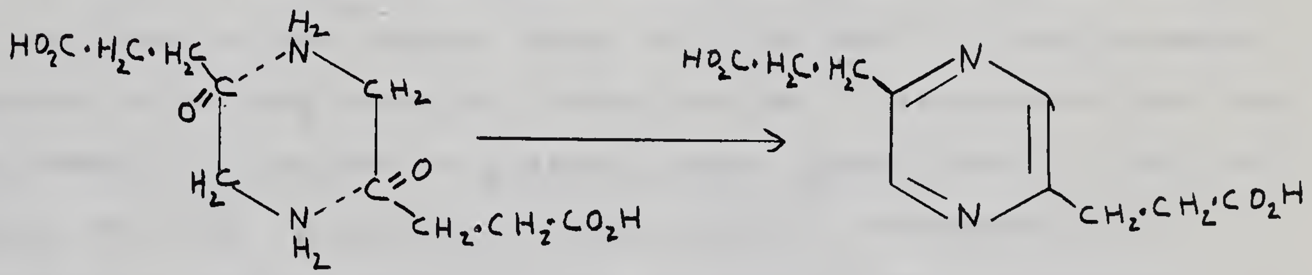




(33) (32)

We have adapted this method for the preparation of pyrazine-3,6-dipropionic acid (34) from two molecules of δ-aminolevulinic acid (35).

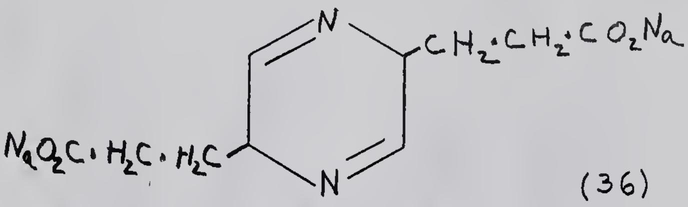


(35) (34)

It was of interest to note that the condensation of δ-aminolevulinic acid to the pyrazine takes place at 37° and pH 7.4 in the presence of air and it was possible to prepare the pyrazine by this means. This indicated that the non-enzymic synthesis of the pyrazine from δ-aminolevulinic acid is possible <u>in vivo</u> if there is a breakdown in cellular antioxidant systems.

(c) SYNTHESIS OF DISODIUM DIHYDROPYRAZINE-3,6-DIPROPIONATE

For the preparation of the dihydropyrazine the method of Scott[25] was used. This consists in adding very strong sodium hydroxide to solid δ-aminolevulinic acid whereupon the dihydropyrazine settles out as the disodium salt (36).



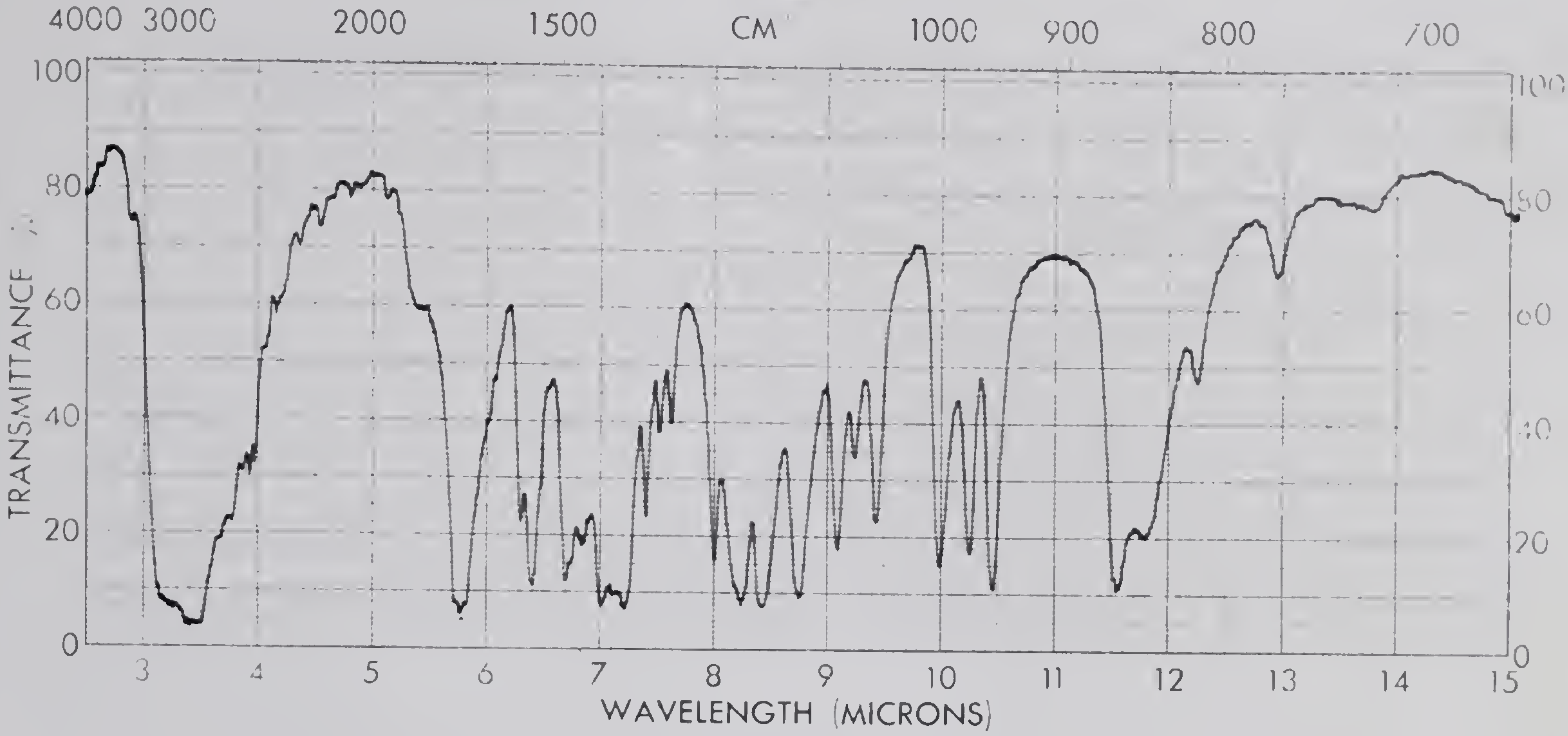
(36)

Due to the lability of this compound we have been unable to obtain it in an analytically pure form as measured by its elemental composition but its infrared spectrum indicated that it was the required dihydropyrazine in accordance with the report of Scott. The spectrum of the dihydropyrazine (figure 5) differs from that of δ-aminolevulinic acid hydrochloride (figure 5) by 1) the loss of the -COOH band at 2500-3000 cm.$^{-1}$, and 2) the loss of the ketone band at 1720 cm.$^{-1}$. Furthermore the absence of an NH_2 band at 3400-3500 cm.$^{-1}$ indicates that the compound does not possess a primary amino group and is thus not the sodium salt of δ-aminolevulinic acid. Furthermore the dihydropyrazine on exposure to air is converted to pyrazine-3,6-dipropionic acid.

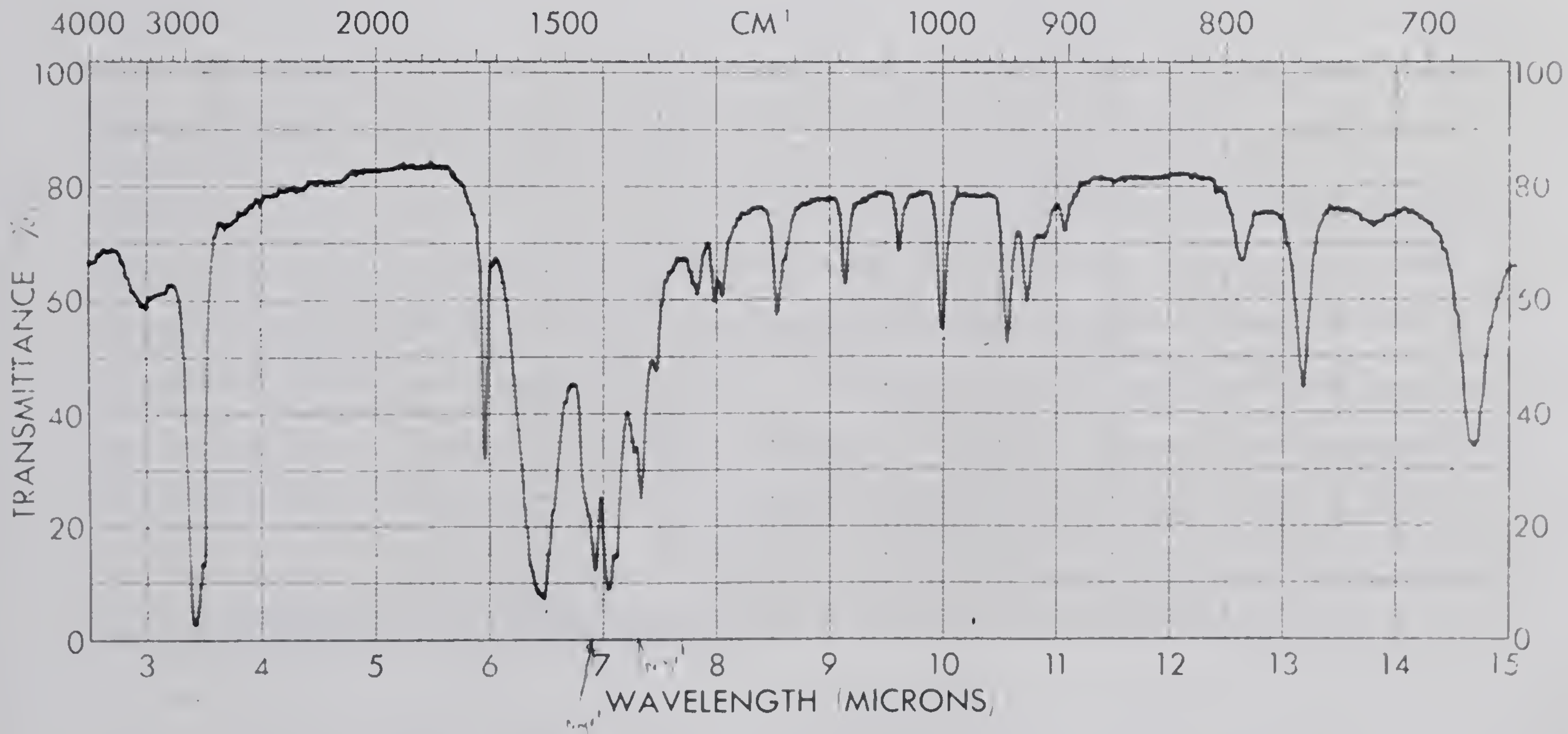
(d) SYNTHESIS OF γ,δ-DIOXOVALERIC ACID

Levulinic acid (37) was converted to β,δ-dibromolevulinic acid (38) by treatment with bromine according to the procedure of Wolff.[26] Mild hydrolysis of the crystalline β,δ-dibromolevulinic acid by boiling in water for four

INFRARED SPECTRA OF δ-AMINOLEVULINIC ACID AND DISODIUM DIHYDROPYRAZINE-3,6-DIPROPIONATE



δ— Aminolevulinic Acid



Disodium Dihydropyrazine -3,6 – Dipropionate

Fig. 5

hours afforded crude γ, δ-dioxovaleric acid (39) which was purified by the procedure of Carpenter.[27]

$$CH_3\text{-}CO\text{-}CH_2\text{-}CH_2\text{-}CO_2H \xrightarrow{Br_2} BrCH_2\text{-}CO\text{-}CHBr\text{-}CH_2\text{-}CO_2H$$

(37) (38)

$$\downarrow$$

$$OHC\text{-}CO\text{-}CH_2\text{-}CH_2\text{-}CO_2H$$

(39)

This acid is very labile and cannot be obtained in a crystalline form. Preparation of a derivative of γ, δ-dioxovaleric acid, viz., the bis p-phenylhydrazone[35] indicated that the purity of the γ, δ-dioxovaleric acid used was approximately 38%. Further purification of this material was achieved by utilizing an ion-exchange chromatographic procedure based on that of Busch, Hurlbert and Potter[37] for the separation of organic acids. The γ, δ-dioxovaleric acid was absorbed on a strong base resin (Dowex 2-X8) in the acetate form and the column washed with water and 1N-acetic acid to remove impurities. The γ, δ-dioxovaleric acid was eluted from the column by increasing the concentration of the acetic acid solution to 3-normal. The yield of bis p-nitrophenylhydrazone prepared from this purified preparation of γ, δ-dioxovaleric acid indicated that a considerable purification had been effected and this material was at least 54% pure. Further purification of the material was considered unnecessary until it could be determined whether the preparation possessed pharmacological activity.

(e) SYNTHESIS OF PORPHOBILIN

When an aqueous solution of porphobilinogen, pH 5.2 is heated for five minutes at 100°, a mixture of uroporphyrin isomers and porphobilin is obtained.[28] A procedure used by Falk[29] for the extraction of uroporphyrins was adapted for the purification of porphobilin. This involves adjusting the pH of the reaction mixture from 5.2 to 1.8 and extracting the

uroporphyrin isomers with cyclohexanone; porphobilin remains in the aqueous phase. Because of the small amount of porphobilinogen available no attempt was made to obtain the porphobilin in crystalline form.

(f) TESTING OF COMPOUNDS FOR PHARMACOLOGICAL ACTIVITY

1. Pyrazine-3,6-dipropionic acid
2. Disodium dihydropyrazine-3,6-dipropionate
3. γ, δ-Dioxovaleric acid
4. Porphobilin

During attacks of acute intermittent porphyria the patient may have hypertension, abdominal pain, constipation and extension of the large gut. For these reasons the compounds were tested on the blood pressure of an anaesthetised cat and on isolated tissues according to the procedures of Goldberg et al.[13] and Jarrett et al.[38] Details of the experimental procedure are recorded in the experimental section.

Animal Experiments

Pyrazine-3,6-dipropionic acid (333 μg./Kg.), γ, δ-dioxovaleric acid (333 μg./Kg.), disodium dihydropyrazine-3,6-dipropionate (333 μg./Kg.) and porphobilin (74 μg./Kg.) when injected into an anaesthetised cat did not alter the blood pressure and neither potentiated nor inhibited the responses to adrenaline or acetylcholine. The results of this experiment are recorded in Table 2.

TABLE 2

Tests of Compounds on Blood Pressure of a Cat* Anaesthetised with Nembutal (30 mg./Kg.)
(Mean blood pressure 138 mm.Hg)

	Acetyl-choline	Adrena-line	Pyrazine-3,6-diprop-ionic acid	Disodium Dihydro-pyrazine-3,6-di-propionate	γ,δ-Dioxo-valeric acid	Por-phobilin
Drug Concen-tration	2 μg/Kg	5 μg/Kg	333 μg/Kg	333 μg/Kg	333 μg/Kg	74 μg/Kg
Effect on Blood Pressure	-25±3 mmHg	+22±2 mmHg	+1±2 mmHg	+3±3 mmHg	+1±2 mmHg	+3±4 mmHg
Effect on Responses to Acetylcholine(a) or Adrenaline(b)			a) +7±1 mmHg b) -2±1 mmHg	b) +2±1 b) -5±2	a)-2±3 mmHg b)-2±5 mmHg	a)-2±2 mmHg b)-2±4 mmHg

0 = no effect; + = increase; - = decrease

Values expressed are mean values ± standard deviation

*Only one cat was used

Isolated Tissues

(Doses are quoted as μg./ml. of bath fluid)

Pyrazine-3,6-dipropionic acid (20 μg./ml.), disodium dihydropyrazine-3,6-dipropionate (20 μg./ml.) and γ, δ-dioxovaleric acid (20 μg./ml.) produced no response and neither potentiated nor inhibited the effects of histamine, acetylcholine or adrenaline on rabbit jejenum or guinea-pig ileum.

Before discussing the results obtained with porphobilin it is necessary to comment on the preparation of this pigment. As previously mentioned porphobilin was prepared from synthetic porphobilinogen. Porphobilinogen has been synthesized by two distinct routes[22,48] so that preparations prepared by different routes could have contained traces of different impurities. In our initial experiments, porphobilin prepared from a sample of synthetic porphobilinogen, obtained from Dr. L. Bogorad, produced a contraction of the guinea-pig ileum which was partially blocked by atropine and mepyramine. In later experiments we prepared porphobilin from a sample of porphobilinogen obtained from Dr. S. F. MacDonald. In these later experiments, porphobilin (28 μg./ml.) produced no response and neither potentiated nor inhibited the effects of acetylcholine or histamine on guinea-pig ileum. It is likely that the differences obtained in the pharmacological testing of porphobilin is the result of utilizing samples of porphobilinogen synthesized by different routes. We attribute our early results to the presence of some impurity in the sample of synthetic porphobilinogen obtained from Dr. L. Bogorad and on the basis of our later results we conclude that it is devoid of pharmacological activity. The results of these experiments are recorded in Table 3.

TABLE 3

Tests of Compounds on Isolated Tissues*

Rabbit Jejenum

	Adrenaline	Acetyl-choline	Histamine	Pyrazine-3,6-dipropionic acid	Disodium Dihydro-pyrazine-3,6-Di-propionate	γ,δ-Dioxovaleric acid	Porphobilin
Drug Concentration in Bath	0.1 μg./ml.	0.1 μg./ml.	0.3 μg./ml.	10 μg./ml.	10 μg./ml.	10 μg./ml.	20 μg./ml.
Effect on Spontaneous Activity or Tone	-28±2 mm	+55±5 mm	No Significant Effect	No Significant Effect	No Significant Effect	No Significant Effect	No Significant Effect
Effect on Response to Histamine, Acetyl-choline or Adrenaline				No Significant Effect	No Significant Effect	No Significant Effect	No Significant Effect

Values expressed are mean values ± standard deviation

*Tissues from 4 rabbits were used

Table 3 continued

TABLE 3 (cont'd)

Tests of Compounds on Isolated Tissues*

		Adrenaline	Acetylcholine	Histamine	Pyrazine-3,6-dipropionic acid	Disodium Dihydropyrazine-3,6-Dipropionate	γ, δ-Dioxovaleric acid	Porphobilin
Guinea-Pig Ileum	Drug Concentration		0.02 μg./ml.	0.05 μg./ml.	20 μg./ml.	20 μg./ml.	20 μg./ml.	28 μg./ml.
	Effect on Tone		+35±2 mm	+23±4 mm	0	0	0	0
	Effect on Response to Acetylcholine or Histamine				No Significant Effect	No Significant Effect	No Significant Effect	No Significant Effect

Values expressed are mean values ± standard deviation
*Tissues from 3 guinea-pigs were used

Summary of Results of Pharmacological Testing

The results of the pharmacological tests carried out indicate that neither the pyrazine-3,6-dipropionic acid, disodium dihydropyrazine-3,6-dipropionate nor γ,δ-dioxovaleric acid are responsible for the hypertension and abdominal pain observed in acute porphyria. Also the tests carried out with porphobilin derived from synthetic porphobilinogen (ex S. F. M.) on guinea-pig ileum indicate that this substance is devoid of intrinsic pharmacological activity, and is not responsible for the abdominal pain observed in acute porphyria. The activity of a porphobilin preparation, isolated from urine, on the guinea-pig ileum is thus shown to be due to contamination with a pharmacologically active substance, as suggested by Goldberg _et al._[13]

The results obtained by Goldberg _et al._[13], Jarrett _et al._[38] and the results reported in this thesis suggest that the various porphyrins and porphyrin metabolites excreted in acute porphyria are not implicated in the symptoms of porphyria. However the possibility cannot be ruled out that the methods used for testing these substances are not sufficiently comprehensive for detecting pharmacological activity. In this connection it is of interest to note that δ-aminolevulinic acid is an ω-amino-n-aliphatic acid closely related in structure to γ-aminobutyric acid (GABA). The latter substance mimics closely the action of the inhibitory nerve upon single stretch receptor neurones and neuromuscular junctions of crayfish. Since a variety of ω-amino-aliphatic acids (homologues of GABA) also possess similar but weaker activity it was of interest to determine whether δ-aminolevulinic acid itself has

[illegible]

[illegible]

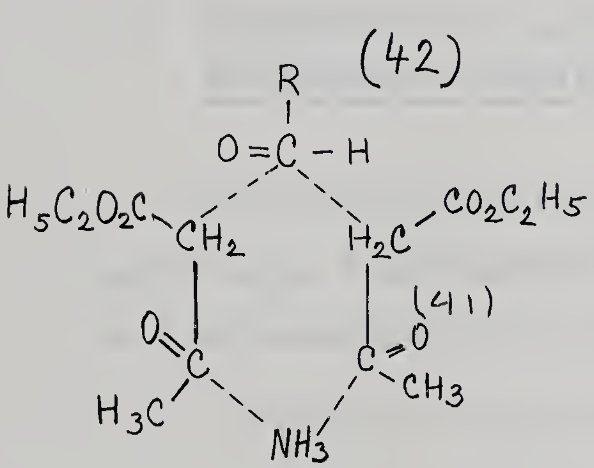
[illegible]

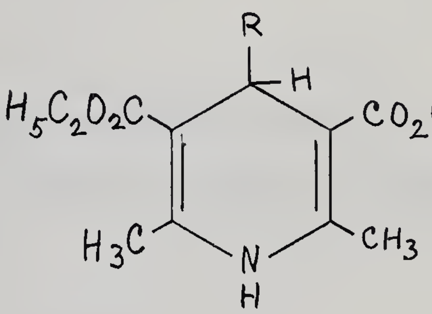
any activity. Tests of the inhibitory action of δ-aminolevulinic acid on the single stretch receptor neurone of the crayfish were carried out by Dr. H. McLennan who reported to us that δ-aminolevulinic acid has about one-fiftieth the activity of GABA. Consequently since porphyrin biosynthesis occurs in all cells it is possible that δ-aminolevulinic acid could be implicated in the neurological symptoms observed in acute porphyria and further testing of this material is warranted.

Part II

(a) SYNTHESIS OF 3,5-DIETHOXYCARBONYL-1,4-DIHYDRO -2,6-DIMETHYL-4-PROPYLPYRIDINE

De Matteis and Prior[30] prepared DDC (40, R=CH_3-) by condensing ethyl acetoacetate (41) and acetaldehyde (42, R=CH_3-) in a solution of ammonium carbonate. We adapted this method for the synthesis of 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-propylpyridine (40, R=$CH_3CH_2CH_2$-) by substituting n-butyraldehyde (42, R=$CH_3CH_2CH_2$-, prepared according to Vogel[31]) for acetaldehyde.

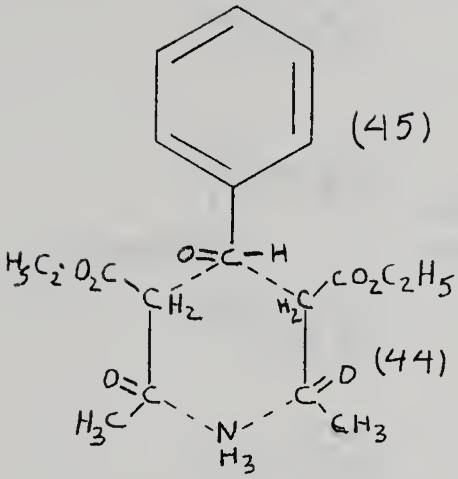




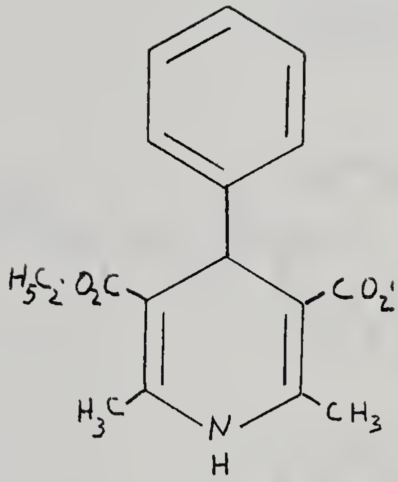
(40)

(b) SYNTHESIS OF 3,5-DIETHOXYCARBONYL-1,4-DIHYDRO -2,6-DIMETHYL-4-PHENYLPYRIDINE (43)

The synthesis employed was that of Traber and Karrer.[32] Dry ammonia gas was passed into a mixture of ethyl aceto-acetate (44) and benzaldehyde (45) in ethanol and the product was heated for four hours under reflux.



(45) (44)



(43)

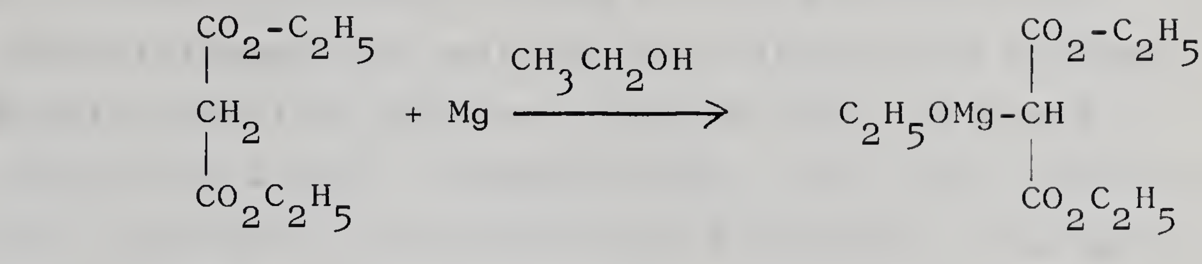
(c) <u>SYNTHESIS OF 3,5-DIETHOXYCARBONYL-1,4-DIHYDRO-2, 6-DIETHYL-4-METHYLPYRIDINE</u>

It was proposed to prepare this compound (46) by condensing acetaldehyde with ethyl propionylacetate (47) in the presence of dry ammonia.

$$CH_3\text{-}CH_2\text{-}CO\text{-}CH_2\text{-}COO\text{-}CH_2\text{-}CH_3 \qquad (47)$$

For this purpose ethyl propionylacetate was required and it was prepared[33] by the following route:- Ethylmagnesiomalonate (49), prepared by the condensation of ethylmalonate (48), magnesium and ethanol, was treated with propionylchloride (50) to yield diethyl n-propionylmalonate (51), which on decarboxylation with β-naphthalene sulfonic acid afforded ethyl propionylacetate (47). 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-diethyl-4-methylpyridine (46) was prepared by passing ammonia gas into a solution of acetaldehyde (52) and ethyl propionylacetate in ethanol

and refluxing the mixture for four hours.



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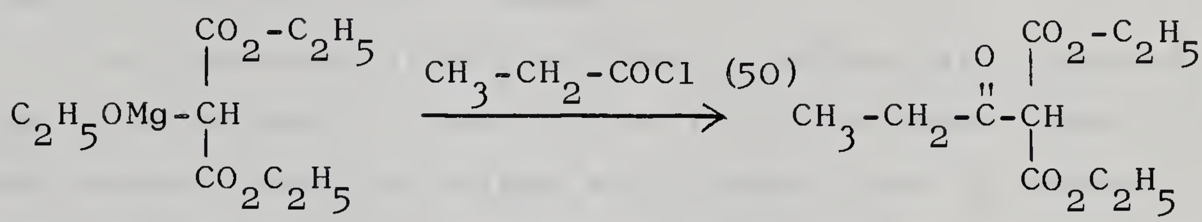

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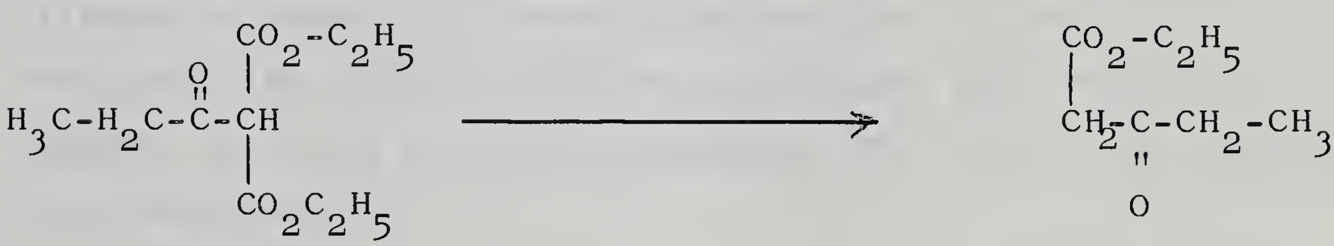

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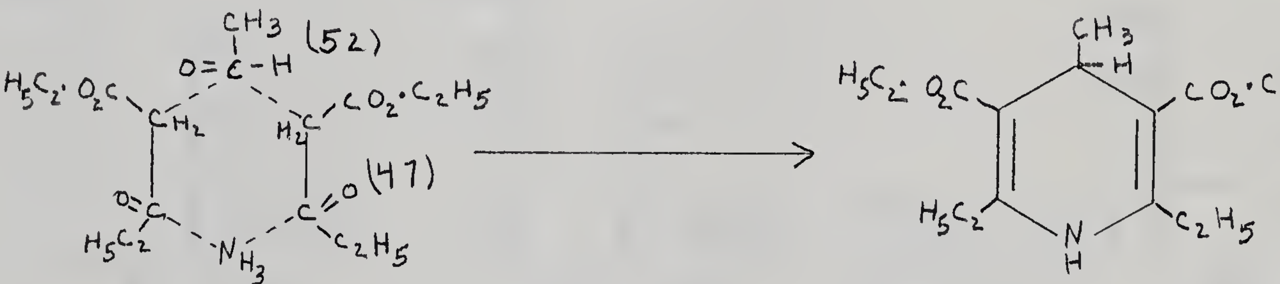


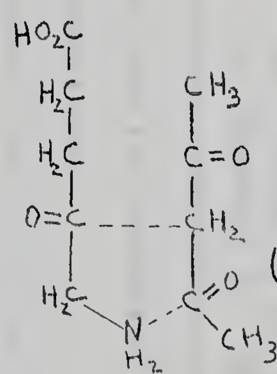
(46)

(d) <u>TESTING OF COMPOUNDS FOR PORPHYRIA-INDUCING ACTIVITY</u>

The ability of the 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) analogues (40, R=propyl) and (43) to induce an increased synthesis of δ-amino-levulinic acid synthetase in guinea-pig liver was determined by feeding these compounds to guinea-pigs and estimating the amount of δ-aminolevulinic acid and porphobilinogen excreted in the urine by the method of Mauzerall and Granick[43]. The method consisted in adding urine from these animals

to a column of Dowex 2 resin in the acetate form. Porphobilinogen was held on the column while urea and δ-aminolevulinic acid were washed from the Dowex 2 column with water. Porphobilinogen was then eluted from the column with acetic acid and determined colorimetrically with Ehrlich's reagent.

The washings from the Dowex 2 column were placed on top of a column of Dowex 50 resin in the acid form. Urea was washed from the column with water, and δ-aminolevulinic acid (53) was eluted with sodium acetate and allowed to react with acetylacetone (54) at pH 4.6; the resulting pyrrole (55) was condensed with Ehrlich's reagent to yield a colored compound (56) which was measured colorimetrically.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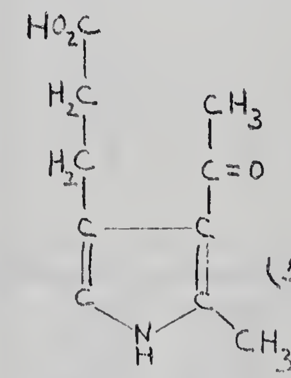
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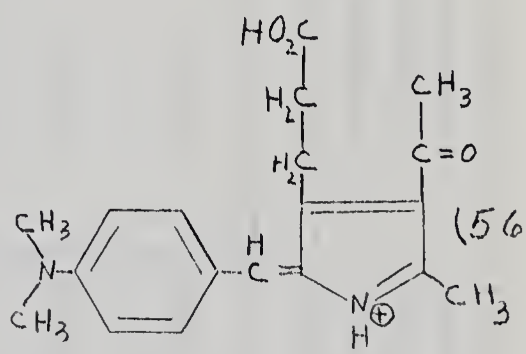

(56)

The results are recorded in Table 4 and in addition the results obtained by previous workers[45] with other DDC analogues (40, R=H) (40, R=CH_3-) and (40, R=CH_3CH_2-) are recorded for comparison. The DDC analogue (46) was not tested by this method due to an insufficient amount of material; instead it was tested in isolated liver cells by a method outlined below.

Studies of the structure activity relationships of porphyria-inducing chemicals in the whole animal suffer from the fact that a distinction cannot be drawn

TABLE 4

Analysis of 24- and 48-Hour Urine of Guinea-pigs Fed DDC or Analogues for 2 Days*

The Extreme Values are Given in Patenthesis

Compound	Number of Animals Used	Average Weight of Animals (g)	Amount of Drug fed per day for 2 days (g)	Component in 24-Hour Urine		Component in 24-48 Hour Urine	
				δ-amino-levulinic acid	Porpho-bilinogen	δ-amino-levulinic acid	Porpho-bilinogen
					μmoles		
40, R=CH_3-CH_2CH_2-	5	662 (600-725)	0.75	1.1 (0.3-3.4)	2.8 (0.4-7.1)	14.4 (0.7-33.4)	25.7 (16.5-49.3)
43	5	324 (316-345)	0.4	0.1 (0.04-0.13)	-	0.1 (0.04-0.13)	-
40, R=H	3	414 (390-425)	0.75	0.8 (0.4-1.1)	-	0.9 (0.3-1.7)	-
40, R=CH_3-	3	354 (326-437)	0.95	0.9 (0.8-0.9)	1.6 (0.2-2.8)	0.9 (0.7-1.3)	1.8 (0.2-3.0)
40, R=CH_3-CH_2-	3	433 (416-496)	0.75	2.1 (0.1-5.0)	3.1 (0.3-5.4)	6.5 (1.6-9.9)	14.6 (10.3-17.7)

40,R=$CH_3CH_2CH_2$- 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-propylpyridine
43 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-phenylpyridine
40,R=H 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine
40,R=CH_3- 3,5-Diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine
40,R=CH_3CH_2- 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine

*In the 0 to 24 hour urine of 3 normal quinea-pigs, δ-aminolevulinic acid 0.1 μM (0.04-0.12) was found; porphobilinogen could not be detected.

between the effects of structural variations at the site of action and on the dynamic phenomena (absorption, distribution, metabolic destruction and excretion) that control drug concentration at that site. This distinction can be drawn by means of a recent technique developed by Granick[7]. The method consists in adding porphyria-inducing chemicals to chick embryo liver cells, cultured on cover slips, and measuring the porphyrin accumulation by means of fluorescence microscopy. The fluorescence intensity was scored as follows: +4, all colonies fluoresce intensely; +3, most colonies fluoresce intensely; +2, all colonies fluoresce partially; +1, most colonies fluoresce partially. In order to eliminate bias in scoring, the samples bore only an identification number and were evaluated independently by two persons. In addition several compounds which had previously been scored by Granick were included to check the accuracy of scoring. The similarity between our two independent scores and the score given by Granick to some of these compounds indicated that our scoring was comparable. From the point of view of a quantitative comparison of the porphyria-inducing activity of these drugs the method has the following limitations: 1) Granick[9] has shown that the fluorescence values of +4, +3, +2, +1 are equivalent, respectively, to 0.5 to 1.0, 0.25 to 0.5, 0.12 to 0.25 and less than 0.1 mμ mole of coproporphyrin per mg. of protein; the method is therefore accurate only within these limits, 2) although Granick has been able, in general, to obtain a normal dose-response relationship, a complication is observed in that high doses of the active drugs appear to cause death of the cells, 3) although the cells are counted and the same number added to each vial, there is no provision for determining the number of living cells at the conclusion of the experiment. In spite of these limitations this method has proved invaluable in demonstrating marked porphyria-inducing activity in compounds thought to be inactive by means of in vivo experiments[45]. Conversely this method may not detect activity in a compound

which is active in vivo by virtue of being metabolized to an active compound. In view of this fact both in vivo and in vitro methods should be employed to determine the activity of porphyria-inducing drugs. Furthermore by this method it is possible to rapidly test a large number of compounds at a wide range of dose levels. The results of testing these analogues (40, R=$CH_3CH_2CH_2$-) and (46) by this method are recorded in Table 5; in addition the DDC analogues (40, R=H), (40, R=CH_3-) and (40, R=CH_3CH_2-) were tested in this system for comparison.

The cultures of chick embryo liver cells to which no porphyria-inducing compounds were added contained a trace of porphyrin (Table 5). Granick[10] has evidence that this trace of porphyrin is produced by some compound present in the diet fed to the hen. By means of careful control of the diet of the hen, porphyrin-free liver cells have been obtained from chick embryos.[10]

As indicated in the introduction we were interested to observe whether the 2,4 and 6-methyl substituents of DDC could be replaced by substituents of similar or larger size, with retention of porphyria-inducing activity. The experiments in quinea-pigs (Table 4) indicated that the order of biological activity was (40, R=$CH_3CH_2CH_2$-) $>$ (40, R=CH_3CH_2-) $>$ (40, R=CH_3-) $>$ (40, R=H) $>$ (43) while the in vitro experiments indicated the following order viz., (46) $>$ (40, R=$CH_3CH_2CH_2$-) and (40, R=CH_3CH_2-) and (40, R=CH_3-) $>$ (40, R=H). The activity of the 4-propyl compound (40, R=$CH_3CH_2CH_2$-) and of the 2,6-diethyl compound (46) is in accord with the view that the substituents at the 2,4 and 6 positions cause a twisting of the ethoxycarbonyl

TABLE 5

CHEMICALS THAT INDUCE PORPHYRIN FORMATION

Fluorescence was scored as follows: +4, all colonies fluoresce intensely; +3, most colonies fluoresce intensely; +2, all colonies fluoresce partially; +1, most colonies fluoresce partially.

Compound Tested	Concentration Molar 10^{-6}	Intensity of Fluorescence 24 Hr. after Addition of Compound
40, R=H	19.7	+0.5
	3.95	trace
	1.97	+0.5
	0.197	trace
40, R=CH_3-	18.7	trace
	3.74	+3.5
	1.67	+0.5
	0.67	trace
40, R=CH_3CH_2-	17.8	+2
	3.56	+2
	1.78	trace
	0.178	trace
40, R=$CH_3CH_2CH_2$-	16.9	+2.5
	3.49	+1.5
	1.74	+1.5
	0.174	trace
46	16.9	+4
	3.49	+3
	1.74	+2
	0.174	trace
no addition		trace

(table 5 cont'd)

TABLE 5 (cont'd)

Compound Tested	Concentration Molar 10^{-6}	Intensity of Fluorescence 24 Hr. after Addition of Compound
95% ethanol (1)		trace
95% ethanol (2)		trace
95% ethanol (3)		trace

40, R=H =3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine

40, $R=CH_3-$ =3,5-Diethoxycarbonyl-1,4-dihydro-2,4,6-timethylpyridine

40, $R=CH_3CH_2-$ =3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine

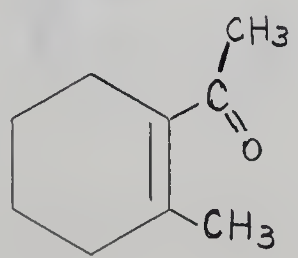
40, $R=CH_3CH_2CH_2-$ =3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-propylpyridine

46 =3,5-Diethoxycarbonyl-1,4-dihydro-2,6-diethyl-4-methylpyridine

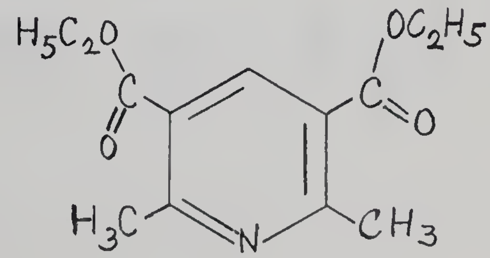
substituents out of the plane of the ring and this non-planar relationship is necessary for optimal activity. The inactivity of the dihydropyridine (43) with a 4-phenyl substituent indicates that the substituents in the 2,4 and 6 positions should be large enough to exert a steric effect on the 3 and 5-ethoxycarbonyl substituents but not so large as to interfere with the fit of the molecule at the receptor.

ULTRAVIOLET ABSORPTION CHARACTERISTICS

As mentioned in the introduction an examination of Fisher-Taylor-Hirschfelder models of compounds (19a and 20a) revealed that because of the presence of the 2 and 6-methyl substituents the introduction of a substituent at position 4 causes a twisting of the ethoxycarbonyl substituents out of the plane of the ring. Further evidence for this molecular configuration was sought by Marks[39] using ultraviolet spectroscopic data. The reasoning was as follows; since 2-methyl acetylcyclohexene (57) exists in the planar s-cis configuration it is probable that the pyridine (58) in which the ethoxycarbonyl substituents are conjugated with the ring will adopt the planar configuration (58). According to Braude and Sondheimer[46], changes in steric conformation in conjugated systems can give rise to two types of spectral effects: 1) changes in absorption intensity alone, 2) wavelength displacement of the characteristic electronic bands.



(57)



(58)

TABLE 6

ULTRAVIOLET CHARACTERISTICS OF DDC AND ANALOGUES

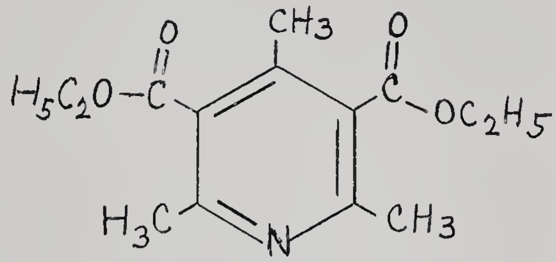
Compound	λ^{*}max.	$\epsilon^{\dagger}$ x 10^{-3}	λmax.	ϵ x 10^{-3}
40, $R=CH_3CH_2CH_2$-	235	16.2	349	7.25
43	239	19.08	356	7.9
46	235	17.8	353	7.8
60	231	16.0	373	7.0
40, $R=CH_3$-	232	18.2	351	8.05
40, $R=CH_3CH_2$-	233	16.0	349	7.41

40, $R=CH_3CH_2CH_2$-	3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-propylpyridine
43	3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-phenylpyridine
46	3,5-Diethoxycarbonyl-1,4-dihydro-2,6-diethyl-4-methylpyridine
60	3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine
40, $R=CH_3$-	3,5-Diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine
40, $R=CH_3CH_2$-	3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine

* λ = wavelength

† ϵ = molecular extinction coefficient

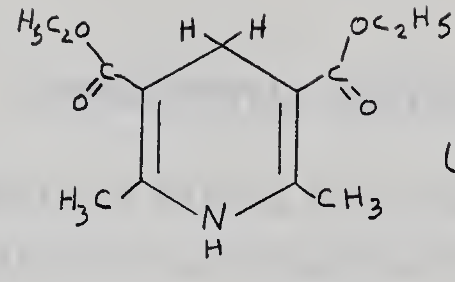
Introduction of a 4-alkyl substituent into the pyridine (58) should result in a twisting of the ethoxycarbonyl substituents out of the plane of the pyridine ring and thereby prevent conjugation of these substituents with the ring. A comparison of the spectra of pyridines (58) and (59) revealed that the introduction of the 4-methyl group had resulted in the anticipated spectral effects mentioned above



(59)

In the present investigation it was of interest to determine in a similar manner to the above whether ultraviolet spectroscopic evidence could provide any insight into the molecular configuration of the dihydropyridines synthesized. For this reason the ultraviolet absorption spectra of compounds (40, R=$CH_3CH_2CH_2$-), (43) and (46) were recorded. In Table 6 these spectra are listed and compared with the spectral data of dihydropyridines previously prepared in our laboratory.

Inspection of the ultraviolet absorption characteristics of these compounds revealed that a shift to lower wavelengths had occurred in one of the absorption bands of the active 4-alkyl substituted dihydropyridines relative to the inactive dihydropyridine (60) which has two hydrogen atoms at the 4-position. This shift to lower wavelengths indicates that a steric inhibition of resonance had occurred in these compounds due to twisting of the ethoxycarbonyl substituents out of the plane of the ring.



(60)

EXPERIMENTAL SECTION

All Melting Points are Uncorrected.
All Analyses were Carried Out by Dr.
C. Daessle, Montreal.

EXPERIMENTAL

ETHYL HYDROGEN SUCCINATE

A mixture of succinic anhydride (100 g., 1 mol.) and ethanol (100 ml., dried with magnesium) was heated on a steam bath. After approximately one hour a clear solution was obtained and heating was then continued for an additional three hours. Excess alcohol was removed in vacuo on a rotary evaporator. The yield was 146 g. (100%).

β-CARBETHOXYPROPIONYL CHLORIDE

A mixture of ethyl hydrogen succinate (146 g., 1 mol.) and thionyl chloride (238 g., 2 mol.) was heated on a water bath at 40-50° for three hours, and excess thionyl chloride removed on a rotary evaporator. Distillation of the residue afforded β-carbethoxypropionyl chloride (139 g., 85%), b.p. 106° /28 mm. Shemin *et al.* record b.p. 98° /15 mm.

ETHYLMAGNESIOMALONATE

In a litre round bottom flask were placed magnesium (18.3 g., 0.75 mol.), carbon tetrachloride (0.725 ml.), dry ethanol (18.3 ml.) and 22 ml. of a mixture of ethyl malonate (118 g., 0.75 mol.) and dry ethanol (57.7 ml.). The remaining ethyl malonate dissolved in ethanol was added through the condenser at such a rate that the reaction proceeds vigorously but not beyond control. When the reaction moderated the flask was cooled and dry ether (225 ml.) added. The crystals which separated out,

dissolved upon gentle heating and the reaction was brought to completion on a steam bath (seven hours). The ether and most of the alcohol were removed by distillation under reduced pressure. To remove the remaining alcohol, benzene (225 ml.) was added to the residue and then removed by distillation under reduced pressure. The ethylmagnesiomalonate residue was dissolved in dry ether (225 ml.).

β-KETO-α-CARBETHOXYADIPATE

Condensation of the ethylmagnesiomalonate (171 g., 0.75 mol.) and β-carbethoxypropionyl chloride (139 g., 0.85 mol.) was accomplished by adding the acid chloride in dry ether to the ethylmagnesiomalonate at such a rate that a vigorous reflux was maintained. After all the acid chloride had been added the reaction mixture was refluxed for two hours. After some time the magnesium complex separated as a brown ether-insoluble viscous mass. The reaction mixture was hydrolyzed by the slow addition of a cold solution of dilute sulfuric acid. The ether layer was separated and the aqueous solution was extracted once with ether. The ether solutions were combined and washed successively with dilute sulfuric acid and water. After drying (sodium sulfate) the ether was removed and the residue distilled under reduced pressure. After collection of a forerun (20 ml.) β-keto-α-carbethoxyadipate (147 g., 72%) was collected as a colorless oil, b.p. 136-138° /0.6 mm. Eisner *et al.*[40] record b.p. 144° /0.2 mm.; Shemin *et al.*[4] record b.p. 125-135° /0.5 mm.

β-KETOADIPIC ACID

To ethyl-β-keto-α-carbethoxyadipate (25 g., 0.358 mol.)

was added concentrated hydrochloric acid (173 ml.) and the mixture kept at room temperature for eighteen hours. Evaporation of the solution to dryness at 30-35° under reduced pressure (rotary evaporator) afforded β-keto-adipic acid as a white solid. It was freed from occluded hydrochloric acid by keeping it in a vacuum dessicator over sodium hydroxide pellets for several days. The product had m.p. 97-100° (decomposition). Eisner *et al.*[40] record m.p. 115° (decomposition).

δ-OXIMINOLEVULINIC ACID

Ethyl nitrite (0.40 mol.) was generated by allowing a solution of concentrated sulfuric acid (2.8 ml.), alcohol (2.8 ml.) and water (27 ml.) to flow into a stirred solution of sodium nitrate (6.6 g.), alcohol (2.8 ml.) and water (27 ml.). The ethyl nitrite gas was passed into a suspension of β-ketoadipic acid (57 g., 0.36 mol.) in glacial acetic acid (125 ml.) maintained at 15-20° for one hour. The crystalline material dissolved on standing at room temperature for one hour and the solution was evaporated to dryness in vacuo at 30-35°. The residue of crude δ-oximinolevulinic acid was used without purification for the next step in the synthesis.

δ-AMINOLEVULINIC ACID

An ice cold solution of stannous chloride dihydrate (62.5 g., 0.28 mol.) in concentrated hydrochloric acid (62.5 ml.) was added to the δ-oximinolevulinic acid and the mixture kept for forty eight hours at 4°. The solution was diluted with water to a volume of 1250 ml.

and hydrogen sulfide gas passed in. The precipitate of tin sulfide was removed by filtration and the filtrate was evaporated to dryness in vacuo at 30-35°. The residue, dried in a dessicator, was triturated with acetone whereupon crystalline material separated (6.5 g.). Recrystallization from methanol-ethyl acetate afforded δ-aminolevulinic acid hydrochloride as white needles, m.p. 150-151° (decomposition). Shemin et al.[4] record m.p. 149-151° (decomposition).

PYRAZINE-3,6-DIPROPIONIC ACID (METHOD I)

δ-Aminolevulinic acid hydrochloride (2.0 g., 0.012 mol.) was dissolved in ammonium hydroxide (4N, 20 ml.) and the solution kept in an open dish overnight. After adjusting the solution to pH 4 with acetic acid a product separated (280 mg.) which after crystallization from methanol yielded pyrazine-3,6-dipropionic acid, m.p. 217-218° (decomposition), λmax 277 mμ (ϵ, 7460), (Found: C, 53.31; H, 5.38; N, 12.16%. $C_{10}H_{12}N_2O_4$ requires C, 53.59; H, 5.40; N, 12.50%).

DIMETHYL ESTER OF PYRAZINE-3,6-DIPROPIONIC ACID

To a solution of pyrazine-3,6-dipropionic acid (0.06 g., 0.0003 mol.) in methanol (6 ml.) was added sulfuric acid (0.1 ml.) and the mixture refluxed for three hours. On cooling, the solution was diluted with sodium carbonate (1%; 28 ml.) and extracted three times with ether (10 ml.). The combined ether extracts were washed successively with sodium carbonate (5%), water, and dried (magnesium sulfate). Removal of the ether afforded the dimethyl ester (0.027 g., 36%) which crystallised from n-heptane as long

white needles m.p. 59-60°. (Found: C, 57.00; H, 6.50; N, 11.65%. $C_{12}H_{16}O_4N_2$ requires C, 57.14; H, 6.45; N, 11.11%).

PYRAZINE-3,6-DIPROPIONIC ACID (METHOD II)

A solution of δ-aminolevulinic acid hydrochloride (25 mg.) in 10 ml. phosphate buffer (pH 7.4;0.2M.) was incubated at 37° for twenty-four hours. Examination of the ultra-violet spectrum and measurement of the height of the absorption band at 277 mμ revealed that 2.1% of the δ-aminolevulinic acid had been converted to pyrazine-3,6-dipropionic acid.

DISODIUM DIHYDROPYRAZINE-3,6-DIPROPIONATE

Treatment of solid δ-aminolevulinic acid hydrochloride (0.5 g., 0.0033 mol.) with saturated sodium hydroxide solution (4 ml.) produced a white precipitate of disodium dihydropyrazine-3,6-dipropionate (0.329 g., 71%) which was collected by centrifugation, washed repeatedly with alcohol and dried in a vacuum desiccator. Although Scott claimed that the nitrogen analysis was in good accord with that required by this compound, we were unable to confirm this observation (required: N, 10.29; found: N, 9.38%). However the infrared spectrum (figure 5) was in agreement with that expected. Further attempts to purify this compound were not undertaken because of its lability and tendency to oxidize.

OXIDATION OF DISODIUM DIHYDROPYRAZINE-3,6-DIPROPIONATE

A solution of disodium dihydropyrazine-3,6-dipropionate (0.329 g., 0.0013 mol.) in water (5 ml.) was exposed

to air for seventy-two hours. Adjustment of the solution to pH 4 with acetic acid and recrystallisation of the precipitate from hot methanol afforded pyrazine-3,6-dipropionic acid (0.046 g., 17%) m.p. 217-218° (decomposition).

β, δ-DIBROMOLEVULINIC ACID

Bromine (24.6 ml., 0.48 mol.) in chloroform (100 ml.) was added dropwise to levulinic acid (redistilled, 25 g., 0.216 mol.) in chloroform (125 ml.) and the mixture stirred mechanically. After the addition of the first few drops of bromine the solution was decolorized by heating. The solution was then cooled in ice and the remaining bromine added at a rate such that the solution remained colorless or pale yellow. Cautious addition of bromine is necessary as the reaction is vigorous and considerable hydrogen bromide is evolved. During the addition of the bromine the β, δ-dibromolevulinic acid begins to crystallise out of the solution. After addition of the bromine, stirring was continued for an additional two hours at 0° and the product (41.8 g., 71%) collected by filtration. It crystallised from dry ether as buff coloured needles, m.p. 113.5-115.5°. Carpenter[27] records m.p. 113-114°.

γ, δ-DIOXOVALERIC ACID

A suspension of β, δ-dibromolevulinic acid (10 g., 0.37 mol.) in water (100 ml.) was heated on a boiling water bath for six hours; a mixture of products was produced, comprised of hydrogen bromide, γ, δ-dioxovaleric acid, diacetyl and uncharacterised substances. Diacetyl which has a low boiling point was removed by distilling the

mixture until 20 ml. of distillate was collected. Most of the hydrogen bromide was removed by stirring the solution with a weak anion exchange resin (Bio-Rad AG3-X4) until the pH rose from 0.6 to 1.8. After separating the resin by filtration and washing it with water the combined filtrate and washings were treated with solid ammonium sulfate (50 g./100 ml.) and extracted ten times with 50 ml. aliquots of ether-ethanol (3:2). The ether-ethanol extracts were combined and dried (magnesium sulfate). Removal of the ether-ethanol in vacuo at 4° afforded a dark brown oil containing γ,δ-dioxovaleric acid.

bis p-NITROPHENYLHYDRAZONE OF γ,δ-DIOXOVALERIC ACID

A solution of p-nitrophenylhydrazine (1.9 g., 0.0125 mol.) in acetic acid (50%, 67.5 ml.) was added to γ,δ-dioxovaleric acid (0.6 g., 0.005 mol.) in water (25 ml.) whereupon a scarlet-red precipitate of bis p-nitrophenylhydrazone formed. The mixture was heated on a boiling water bath for two hours to ensure completion of the reaction. The precipitate crystallised from hot nitrobenzene as orange-red needles which after washing successively with alcohol and ether had m.p. 258-259° (decomposition). Viebel[35] records m.p. 270-271° (decompositon).

PURIFICATION OF γ,δ-DIOXOVALERIC ACID

A solution of the oil containing γ,δ-dioxovaleric acid (0.2 g.) in water (10 ml.) was placed on a column of Dowex 2-X8 (acetate form) and the column washed successively with water (2 x 15 ml.) and acetic acid (1N, 15 ml.). The γ,δ-dioxovaleric acid was eluted with acetic acid

(3N, 2 x 15 ml.) and the acetic acid removed in vacuo (rotary evaporator). The bis p-phenylhydrazone (0.106 g., 54%) was prepared from this purified material by the method described above.

SYNTHESIS OF PORPHOBILIN

Synthetic porphobilinogen (4.2 mg.) was dissolved in 0.002 M.NaOH (5 ml.) and the pH of the solution adjusted to 5.2 with 0.01 M.HCl. The mixture was heated at 100° for five minutes and after cooling the pH was adjusted to 7.4. The reaction mixture gave a negative Ehrlich reaction indicating that all the porphobilinogen was consumed. Examination of the visible spectrum of this solution (figure 6) revealed the presence of porphobilin (λmax 484 mμ) and uroporphyrin (λmax 402 mμ). A part of this solution (1 ml.) was adjusted to isotonicity by adding sodium chloride (9 mg.) and used for pharmacological testing immediately after preparation. The remainder of the solution was adjusted to pH 1.8 with 1 M. HCl and extracted with cyclohexanone. The uroporphyrin together with some porphobilin passed into the cyclohexanone (figure 7a) and porphobilin together with a trace of porphyrin remained in the aqueous phase. The aqueous phase (figure 7b) was adjusted to pH 7.4, adjusted to isotonicity and used for pharmacological testing.

CALCULATION OF THE CONCENTRATION OF PORPHOBILIN

Since porphobilin has not yet been isolated in the pure crystalline form its molecular extinction coefficient is unknown. The following method was used to calculate an approximate value of the molecular extinction coefficient for measuring the concentration of porphobilin solutions.

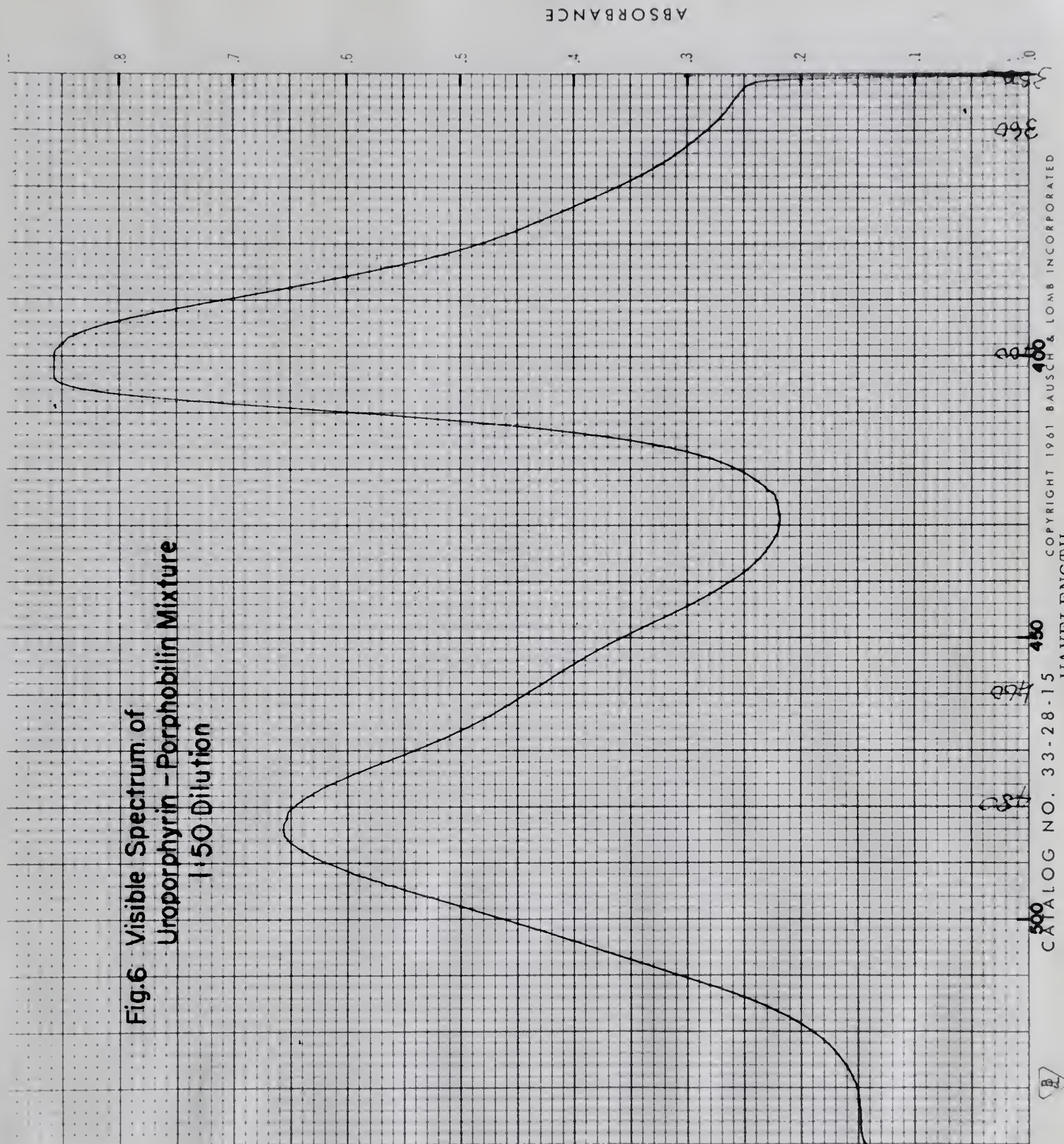


Fig.6 Visible Spectrum of Uroporphyrin - Porphobilin Mixture 1:50 Dilution

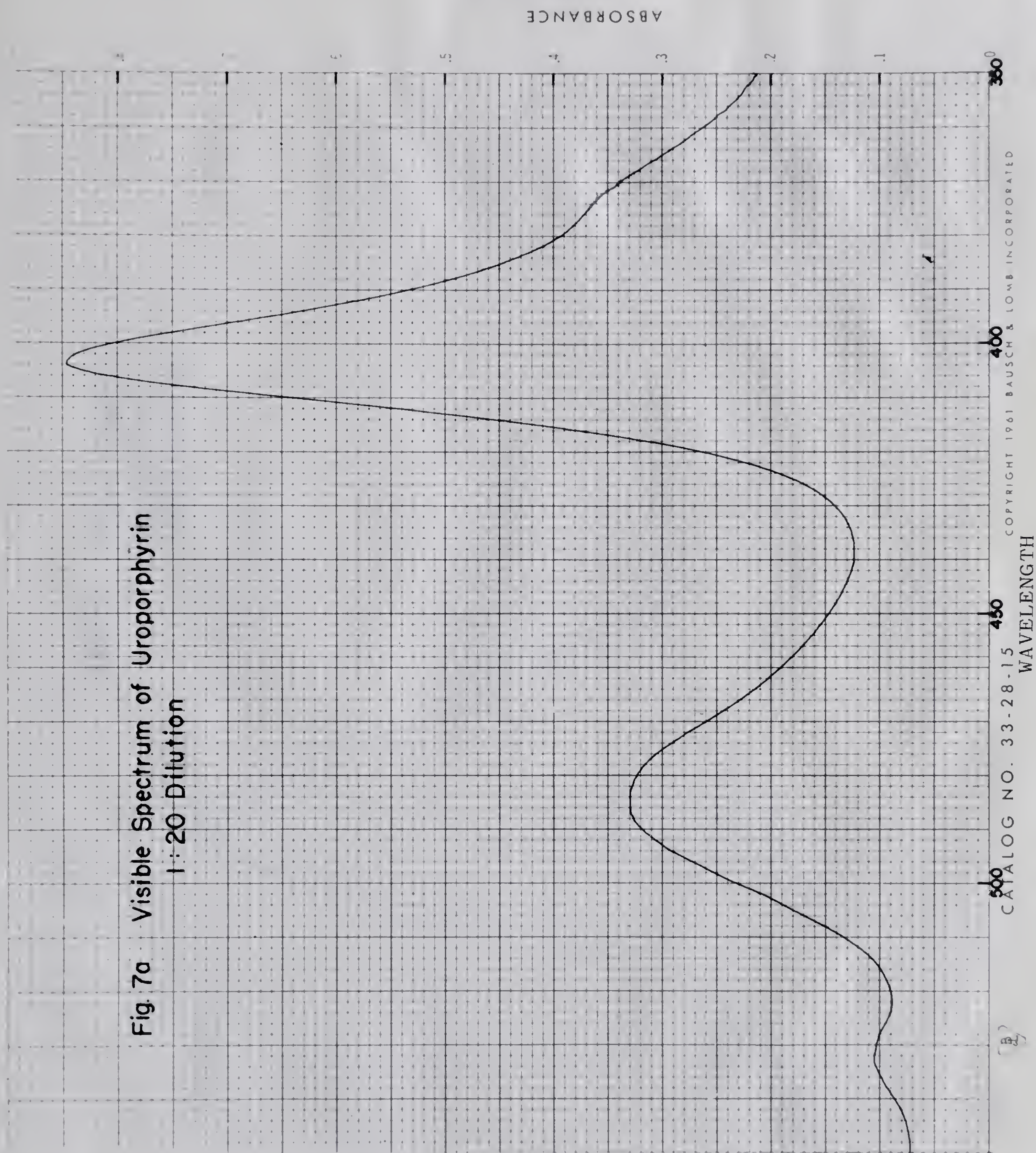


Fig. 7a Visible Spectrum of Uroporphyrin
1:20 Dilution

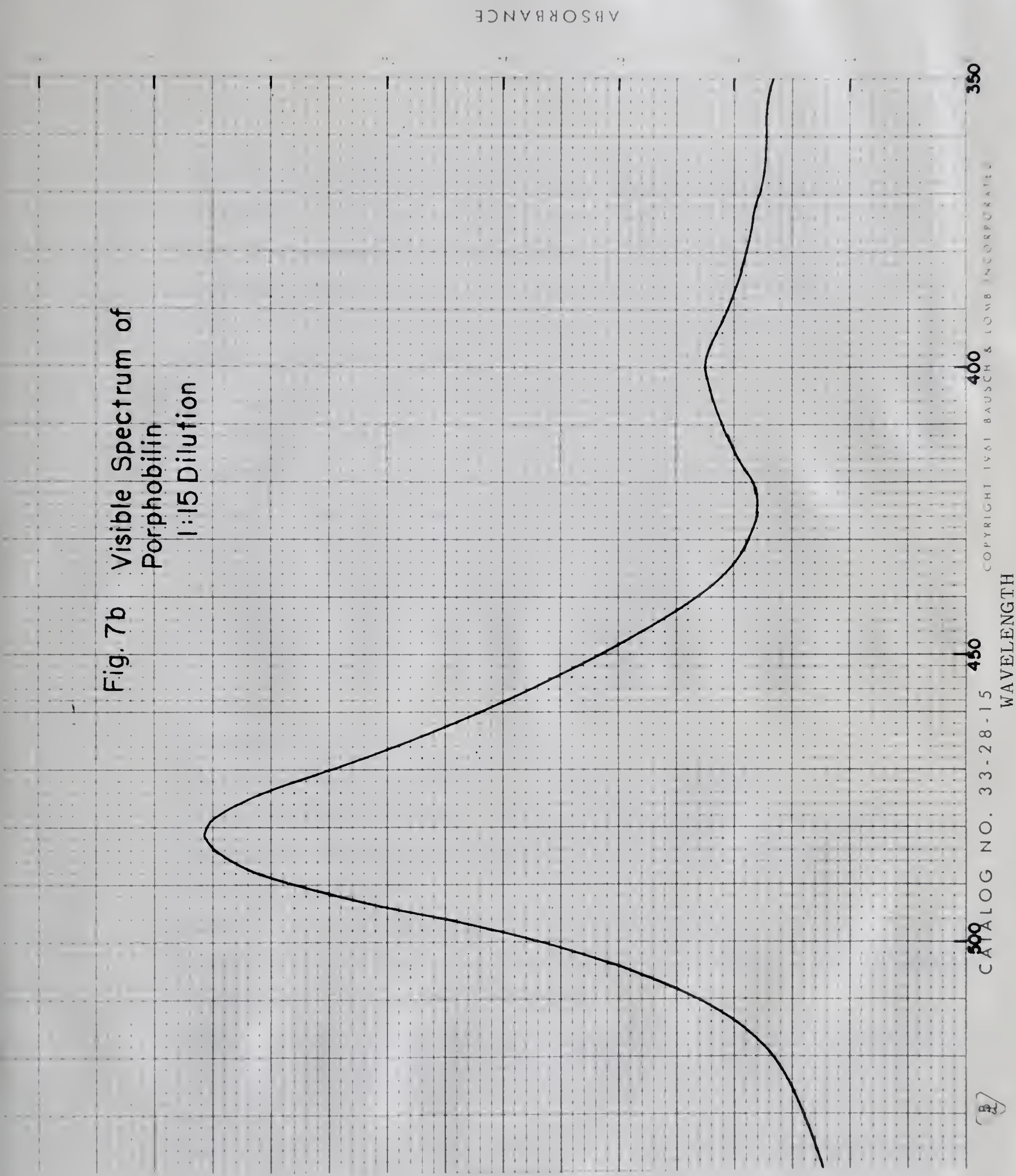


Fig. 7b Visible Spectrum of Porphobilin 1:15 Dilution

Following the conversion of porphobilinogen to a mixture of uroporphyrin isomers and porphobilin (figure 7a), the concentration of uroporphyrin was calculated from the previously recorded molecular extinction coefficient[47] (Soret band; ϵ,106,000). Since one mole of uroporphyrin is derived from four moles of porphobilinogen the amount of porphobilinogen utilized for uroporphyrin formation could be calculated. This value was subtracted from the initial porphobilinogen concentration and it was assumed that the remaining porphobilinogen had been converted to porphobilin. On the further assumption that two moles of porphobilinogen condense to form one mole of porphobilin an approximate molecular extinction coefficient can be calculated. Details of this calculation are as follows:

Volume of solution = 5.25 ml.

Initial porphobilinogen: 4.2 mg. = 18.6 μM

Final porphobilinogen: 0 mg. (negative Ehrlich test)

Molecular extinction coefficient of uroporphyrin: λ max 402 mμ, ϵ,106,00

Amount of uroporphyrin: 2.14 μM/5.25 ml.

Number of moles of porphobilinogen utilised in uroporphyrin formation: 4 x 2.14 = 8.56 μM

Number of moles of porphobilinogen utilised for porphobilin formation = Initial porphobilinogen - porphobilinogen utilised in uroporphyrin formation. (18.6 μM - 8.56 μM = 9.04 μM)

Number of moles of porphobilin: 9.04 $\div$ 2 = 4.52 μM/5.25 ml.

Prepared a 1: 50 dilution (0.0173 μM/ml.) which had an optical density of 0.65

Therefore molecular extinction coefficient is 0.65 x 1000 $\div$ 0.0173 = 37,600.

TESTING OF COMPOUNDS FOR PHARMACOLOGICAL ACTIVITY

1. Pyrazine-3,6-dipropionic acid
2. Disodium dihydropyrazine-3,6-dipropionate
3. γ,δ-Dioxovaleric acid
4. Porphobilin

Animal Experiments

A three kilogram female cat was anaesthetised by intraperitoneal injection of pentabarbital sodium (30 mg./Kg.). The blood pressure was recorded by means of a smoked drum and a mercury manometer. All drugs tested were injected as solutions in isotonic sodium chloride and followed with isotonic saline (2 ml.).

Isolated Tissues

The tissues used in these experiments were rabbit jejenum and guinea-pig ileum. The animals were killed by a blow on the head and the tissues were rapidly removed and rinsed with cold physiological salt solution equilibrated with a mixture of 95% oxygen and 5% carbon dioxide. For isotonic recording the segment of intestine (approximately 1.5 cm.) was secured at both ends with hooks and mounted vertically in a tissue bath containing 10 ml. of physiological salt solution which was equilibrated with a mixture of 95% oxygen and 5% carbon dioxide and maintained at 37°. The tissues were allowed to recover in the bath for one-half to three-quarters of an hour before experiments were begun.

Recording Devices

Isotonic recording was by means of a frontal writing lever of amplification 14:1 and a smoked drum.

Drugs and Solutions

Fresh solutions of drugs in isotonic sodium chloride were prepared prior to each experiment. The modified Krebs-Ringer solution used in testing rabbit jejenum had the following composition in milliequivalents per liter: Na, 138.5; K, 4.6; Ca, 4.9, Mg, 2.3; HCO_3, 21.91; PO_4, 3.48; SO_4, 2.32; glucose, 50. Guinea-pig ileum was maintained in Tyrode's solution which had the following composition in milliequivalents per liter: Na, 150.2; K, 2.68; Ca, 3.61; Mg, 0.21;HCO_3, 43.5; PO_4, 1.25; glucose, 5.55. The solutions were equilibrated with a mixture of 95% oxygen and 5% carbon dioxide.

Procedure

The tissues were exposed to the drug for periods of thirty seconds at two minute intervals.

3,5-DIETHOXYCARBONYL-1,4-DIHYDRO-2,6-DIMETHYL-4-PROPYLPYRIDINE

A mixture of ethyl acetoacetate (33.3 ml., 0.252 mol.) and n-butyraldehyde (11.5 ml., 0.126 mol.) in aqueous ammonium carbonate (10%, 500 ml.) was stirred and kept at 4° for twelve hours whereupon a yellow oil formed. Removal of the yellow impurities from this oil by stirring for five hours with hydrochloric acid (3N., 200 ml.) afforded crystalline 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-propylpyridine (14.2 g., 40%). After

recrystallising three times from ethanol-water, the product had, m.p. 116-120°, λ max 235 and 349 mμ (ϵ, 16,200 and 7250)(figure 8b). Infrared (Nujol mull): Max. 3350 cm.$^{-1}$ (NH dihydropyridine), 1700 cm.$^{-1}$ (conjugated carbonyl) (figure 8a). Jaeckle[41] records m.p, 118°.

3,5-DIETHOXYCARBONYL-1,4-DIHYDRO-2,6-DIMETHYL-4-PHENYLPYRIDINE

Dry ammonia gas was passed into a solution of benzaldehyde (20 g., 0.188 mol.) and ethyl acetoacetate (49 g., 0.378 mol.) in alcohol (100 ml.) for one hour at room temperature. The reaction mixture was heated on a boiling water bath for four hours and the alcohol was removed by distillation whereupon the product (42 g., 68%) crystallised out. Three recrystallisations from hot alcohol afforded 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-phenylpyridine as pale yellow needles, m.p. 156.5-157.5°, λ max 239 and 356 mμ (ϵ, 19080 and 7900)(figure 9b). Infrared (Nujol mull): Max. 3350 cm.$^{-1}$ (NH dihydropyridine), 1700 cm.$^{-1}$ (conjugated carbonyl)(figure 9a). Traber and Karrer[32] record m.p. 156-157°.

DIETHYL n-PROPIONYLMALONATE

Propionyl chloride (49 g., 0.53 mol.) in dry ether (50 ml.) was added to ethylmagnesiomalonate (122 g., 0.5 mol.) (prepared as described above) and the reaction mixture refluxed for thirty minutes. Dilute sulfuric acid (75 ml., 0.25 N.) was added and the mixture stirred. The ether layer was separated and washed successively with dilute sulfuric acid and water. After drying (sodium sulfate) the ether was removed and the residue distilled. After collection of a forerun the product

ULTRAVIOLET AND INFRARED SPECTRA OF 3,5-DIETHOXYCARBONYL-1,4-DIHYDRO-2,6-DIMETHYL-4-PROPYLPYRIDINE

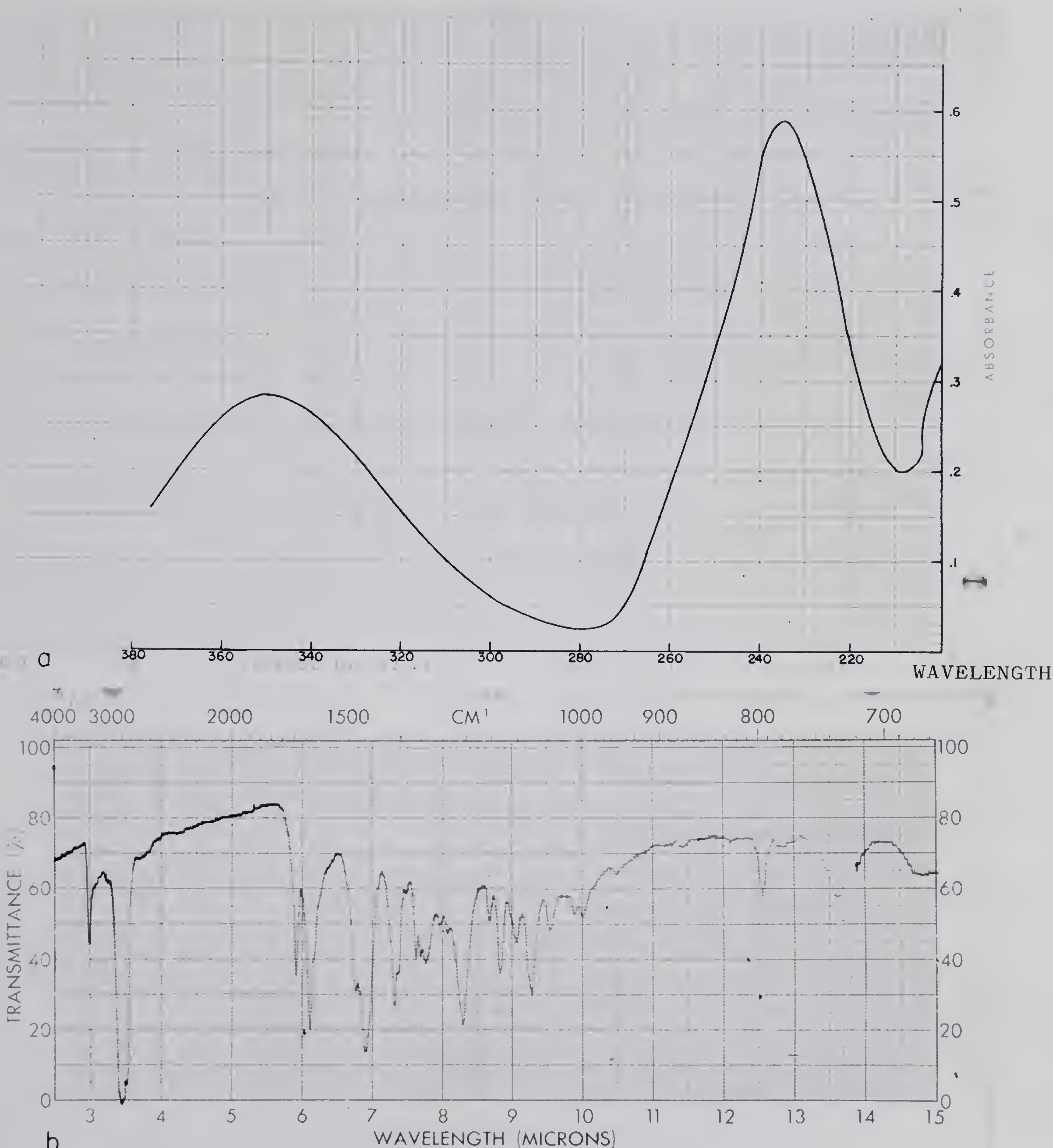
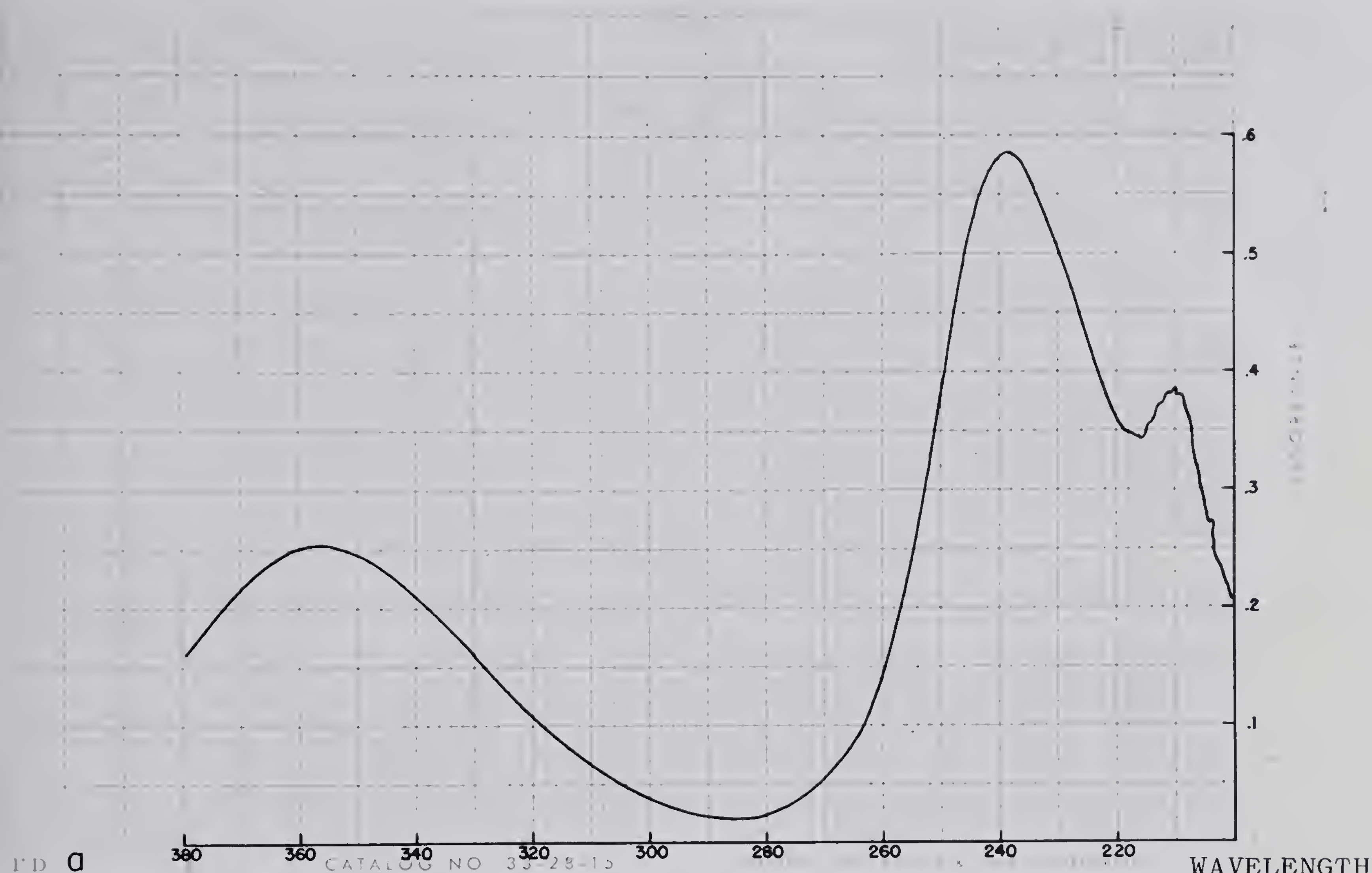


Fig. 8

3,5- Diethoxycorbonyl -1,4- Dihydro -2,6- Dimethyl -4- Propylpyridine

ULTRAVIOLET AND INFRARED SPECTRA OF 3,5-DIETHOXYCARBONYL-1,4-DIHYDRO-2,6-DIMETHYL-4-PHENYLPYRIDINE



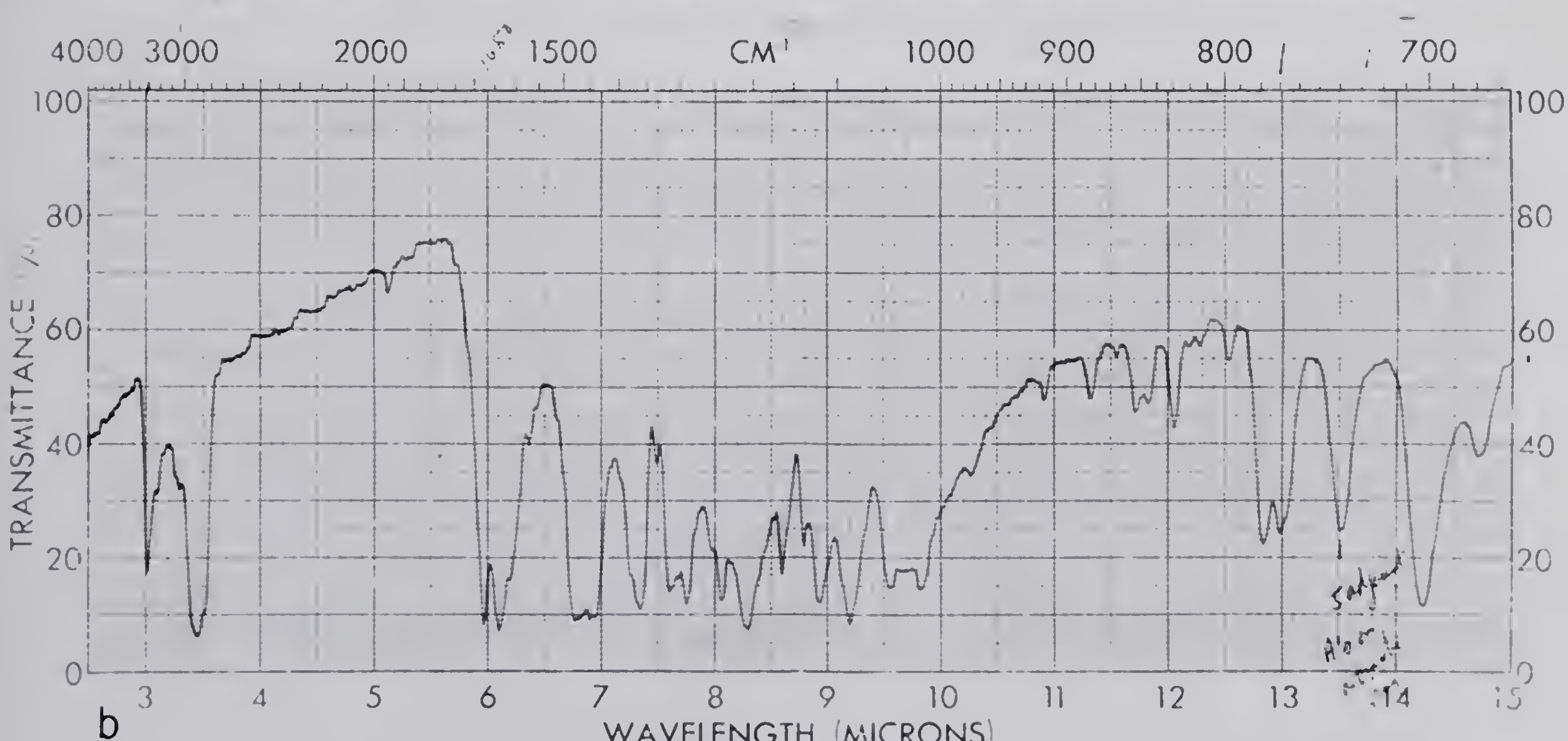


Fig. 9

3,5 - Diethoxycarbonyl - 1,4 - Dihydro - 2,6 - Dimethyl - 4 - Phenylpyridine

was obtained as a colorless oil, b.p. 75-81°/0.38 mm.

ETHYL PROPIONYLACETATE

A mixture of β-naphthalene sulfonic acid (8 g.) and diethyl n-propionylmalonate (49 g., 0.53 mol.) was slowly heated to 200° and maintained at this temperature until evolution of gas ceased (ten hours). The mixture was cooled, diluted with ether and washed repeatedly with dilute sodium carbonate and finally with water. The aqueous washings were extracted with ether and the ether solutions combined. After drying (sodium sulfate) and removing the ether, the residue was distilled through an 18 inch electrically heated column, packed with Fenske rings. Ethyl propionylacetate was obtained as a colorless oil b.p. 87-89°/15.5 mm. Riegel and Lilienfeld[33] record b.p. 100-108°/22 mm., Anderson *et al.*[42] record b.p. 77-77.5°/8.5 mm.

2-ETHYL-3-ETHOXYCARBONYL-4-(3-PROPIONIC ACID) PYRROLE

A solution of δ-aminolevulinic acid hydrochloride (0.344 g., 0.002 mol.) and ethyl propionylacetate (0.144 ml., 0.001 mol.) in 50 ml. of phosphate buffer (pH 6.6, 0.25 M.) was refluxed for fifteen minutes, adjusted to pH 7 and extracted with chloroform. The aqueous layer was separated and aerated to remove traces of chloroform. After adjusting the solution to pH 1, 2-ethyl-3-ethoxycarbonyl-4-(3-propionic acid) pyrrole separated as white crystals which were recrystallised from methanol-water, m.p. 154.5-156° (Found: C, 60.10; H, 6.98; N, 5.83%; $C_{12}H_{17}O_4N$ requires C, 60.23; H, 7.19; N, 5.86%).

3,5-DIETHOXYCARBONYL-1,4-DIHYDRO-2,6-DIETHYL-4-METHYLPYRIDINE

Dry ammonia gas was passed into a solution of acetaldehyde (1.14 ml., 0.021 mol.) and ethyl propionylacetate (6 ml., 0.042 mol.) in alcohol (15 ml.) for one-half hour and the solution was kept at room temperature for an additional twelve hours. After heating on a boiling water bath for two hours and removing the alcohol, the residue was diluted with ether and freed of a yellow impurity by washing successively with hydrochloric acid (3N.) and water. After drying the ether solution (magnesium sulfate), the ether was removed and the crude 3,5-diethoxycarbonyl-1,4-dihydro-2,6-diethyl-4-methylpyridine (0.645 g., 10.5%) was recrystallised from n-heptane as long white needles m.p. 111-112.5°, λ max 235 and 353 mμ (ϵ, 17,800 and 7,800) (figure 10b). Infrared (Nujol mull): Max. 3350 cm.$^{-1}$ (NH dihydropyridine), 1700 cm.$^{-1}$ (conjugated carbonyl). (Found: C, 65.05; H, 7.99%; $C_{16}H_{25}O_4N$ requires C, 65.06; H, 8.53%). Infrared spectrum, figure 10a.

TESTING OF COMPOUNDS FOR PORPHYRIA-INDUCING ACTIVITY IN GUINEA-PIGS

Male guinea-pigs were placed in metabolic cages allowing separate collection of urine and faeces. To induce a porphyria the animals were deprived of food on the first day and on the following two days were fed the drug under investigation once daily by gastric intubation. Solid drugs were suspended in 0.25 M sucrose (1 gm./12.5 ml.) for intubation. The dose of drug and weight of animals used in each experiment are recorded in table 4. Urine samples were collected at twenty-four and forty-eight hours and were frozen until analyzed.

ULTRAVIOLET AND INFRARED SPECTRA OF 3,5-DIETHOXYCARBONYL-1,4-DIHYDRO-2,6-DIETHYL-4-METHYLPYRIDINE

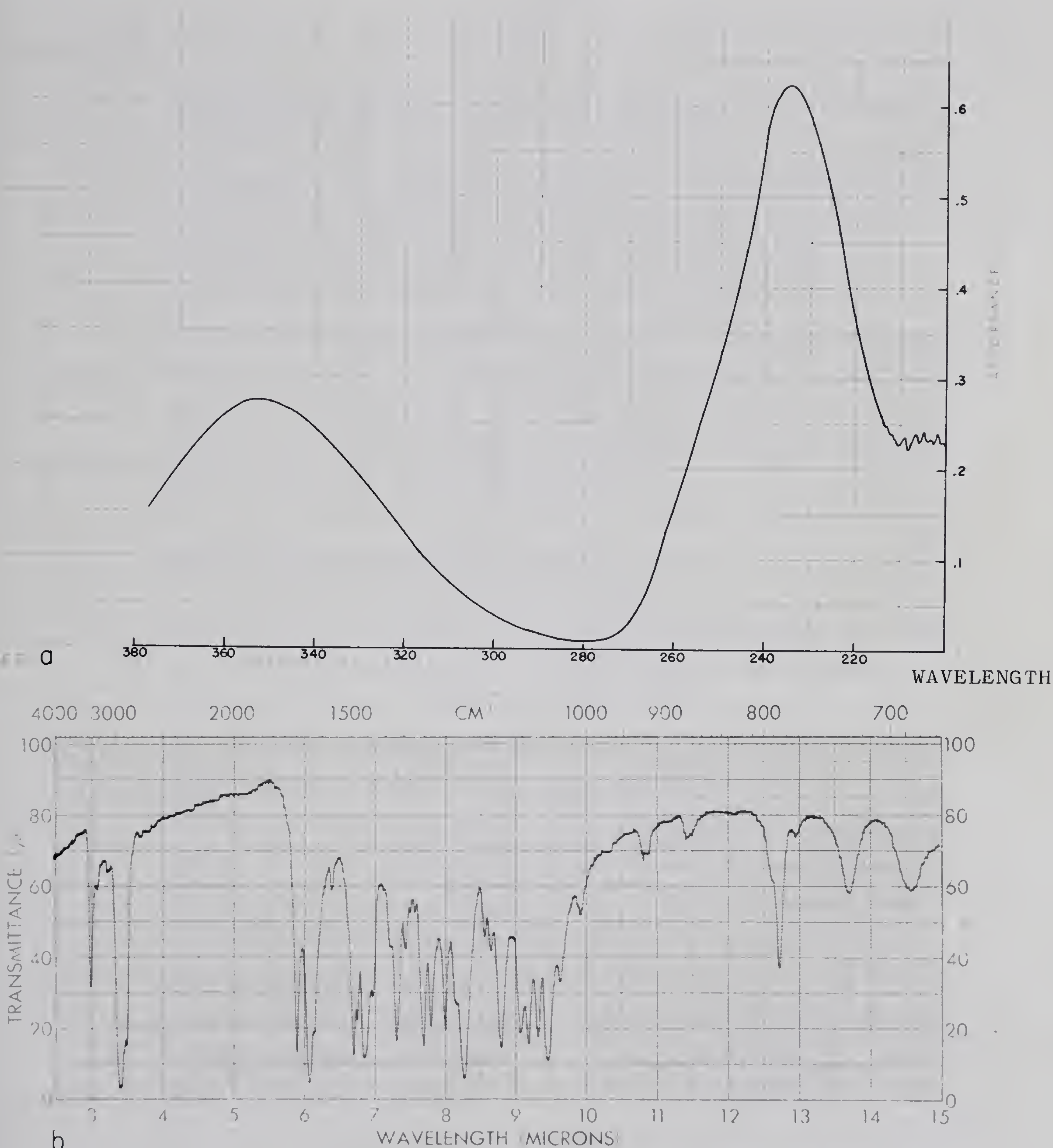


Fig. 10

3,5 - Diethoxycarbonyl -1,4 - Dihydro -2,6- Diethyl -4- Methylpyridine

COLORIMETRIC DETERMINATIONS AND REAGENTS

Colorimetric measurements were made at room temperature with a Unicam SP 600 spectrophotometer in cells of one centimeter light path. Modified Ehrlich's reagent 2 N with respect to perchloric acid was prepared as follows: p-dimethylaminobenzaldehyde (1 g.) was dissolved in glacial acetic acid (30 ml.), perchloric acid (8.0 ml., 70%) was added and the solution was diluted to fifty ml. with acetic acid. The reagent was somewhat unstable and was used on the day it was prepared.

PREPARATION OF RESINS AND COLUMNS

The resins were commercial samples of 200-400 mesh. They were placed in water and decanted until the supernatant fluid was clear. The Dowex 2-X8 resin was converted to the acetate form by washing the resin on a column with 3 N sodium acetate until the eluate was chloride-free. It was then washed with water until the eluate was free of sodium acetate. The Dowex 50-X8 was converted first to the sodium form by allowing it to stand overnight with 2 N sodium hydroxide; it was then washed until neutral and reconverted to the acid form by treating it with about one volume of 4 N-HCl, then in turn with six volumes of 2 N-HCl, 1 N-HCl and water. The columns were 0.7 x 30 cm. tubes with indentations at a level 10 cm. from the lower end. This lower end was made water-repellent with Beckman Desicote. A glass wool plug was placed above the indentations and a slurry of resin sufficient to give 2.0$\pm$0.1 cm. of settled material was added. A glass wool plug on the top completed the columns.

BUFFERS

Acetate buffer of pH 4.6 was made by adding glacial acetic acid (57 ml., 1 mol.) to sodium acetate (136 g., 1 mol.) and diluting to one litre.

DETERMINATION OF PORPHOBILINOGEN AND δ-AMINOLEVULINIC ACID IN URINE

An aliquot of urine (1 ml.) adjusted to pH 5-7 was placed on a column of Dowex 2. Two portions of water (2 ml.) were then added. The combined eluate was quantitatively transferred to the Dowex 50 column. The porphobilinogen was eluted from the Dowex 2 column by adding 1 M- acetic acid (2 ml.), allowing it to drain, and then adding 0.2 M- acetic acid (2 ml.). The combined eluates were quantitatively transferred to a ten ml. volumetric flask and diluted to the mark with water. Modified Ehrlich's reagent (2 ml.) was added to an aliquot of the porphobilinogen solution (2 ml.). The mixture was placed in a cuvette of one centimeter path and the optical density at 553 mμ determined fifteen minutes after mixing. A solution of equal volumes of Ehrlich's reagent and water was used as a blank. The concentration of porphobilinogen was calculated from the previously determined molecular extinction coefficient (6.1×10^4).

The Dowex 50 column containing δ-aminolevulinic acid and urea was washed with water (16 ml.) to remove the urea. Then 0.5 M- sodium acetate (3 ml.) was added. After draining, the δ-aminolevulinic acid was eluted by the addition to the column of 0.5 M-sodium acetate (7 ml.). This eluate was collected in a ten ml. volumetric flask, acetylacetone was added (0.2 ml.), and the solution was

diluted to the mark with acetate buffer pH 4.6. The stoppered flask was placed in boiling water for ten minutes, then cooled to room temperature. To an aliquot of this solution (2 ml.) was added modified Ehrlich's reagent (2 ml.); the solution was mixed and placed in a cell of one centimeter light path. After fifteen minutes the optical density at 553 mμ was read against a blank. The blank consisted of water treated in the same manner as the Dowex 50 eluate. The concentration of δ-aminolevulinic acid was calculated from the previously determined molecular extinction coefficient (7.2×10^4).

The results of this study are reported in Table 4.

TESTING OF COMPOUNDS FOR PORPHYRIA-INDUCING ACTIVITY IN CULTURES OF CHICK EMBRYO LIVER CELLS

(all procedures in this experiment were carried out using standard microbiological aseptic technique)

Preparation of Reagents

a) Calcium and Magnesium-free Earle's Medium

This medium contained the following substances dissolved in one litre of water and adjusted to pH 6.8 for storage.

NaCl	6.8 g.
KCl	0.4 g.
$NaH_2PO_4 \cdot H_2O$	0.125 g.
Dextrose	1.0 g.
$NaHCO_3$	2.2 g.

The solution was sterilized by filtration through a Millipore filter (porosity 0.45μ.).

b) Pangestin Solution

One gram of Pangestin (1:75 Difco certified), which is an active preparation of the enzymes of the pancreas (principally amylopsin, trypsin and steapsin) was added to calcium and magnesium-free Earle's medium (100 ml.) and allowed to stand for twelve hours. The pH was adjusted to 6.8 and the suspension filtered by gravity. The solution was finally filtered through a Millipore filter at 4°. The filtrate was divided into 5 ml. aliquots which were stored in screw cap culture tubes at -15°.

c) Medium for Culturing Liver Cells

The following compounds were added to a 150 ml Pyrex screw cap bottle:

(i) 100 Ml. Eagle's Basal Medium without phenol red (Microbiological Associates, cat. no. 12-104).

(ii) 1 Ml. glutamine solution (Microbiological Associates)(this solution is kept at -15° until used).

(iii) 10,000 U. of buffered penicillin G (crystalline potassium salt, Eli Lilly and Co.) dissolved in 0.25 ml. water.

(iv) 10 Mg. streptomycin sulfate (U.S.P. Eli Lilly and Co.) dissolved in 0.25 ml. water.

(v) 2,000 U. mycostatin (Squibb) dissolved in 0.25 ml. water.

(vi) 10 Ml. Fetal Bovine Serum (Microbiological Associates, cat. no. 14-413).

d) Preparation of Enzyme Mixture for Digestion of Cells

Calcium and magnesium-free Earle's medium (6 ml.) was added to a vial containing sterile, crystallised and lyophilised trypsin (100 mg.). 3 Ml. of Pangestin solution were added to the vial and the contents of the vial mixed whereupon a clear solution resulted. This enzyme mixture was prepared following the removal of the liver from the chick embryo and was always used immediately after preparation.

PREPARATION OF LIVER CELLS

The liver from a seventeen day old chick embryo was washed with calcium and magnesium-free Earle's medium in a small petri dish. The liver was transferred to a second petri dish containing the enzyme mixture, cut up into small pieces with a razor blade and a small amount of the enzyme Varidase was added on the tip of a spatula. After incubating the mixture on a serological bath at 37° until most of the cells were separated (15-30 mins.), the suspension was transferred to a conical centrifuge tube whereupon the larger particles settled to the bottom of the tube. Aliquots (0.05 ml.) of the supernatant suspension of free cells were innoculated into aluminum capped culture vials (O.D. 19 x 63 mm.) containing a glass cover slip (size 5/8 inch, thinness 0) and 1 ml. of culture medium. The cells were incubated at 37° in an atmosphere of moist 95% air:5% carbon dioxide for twenty-four hours.

ADDITION OF CHEMICALS TO LIVER CELLS

The culture medium was removed from each culture vial by means of a Pasteur pipette and replaced with fresh medium (1 ml.). The chemicals under investigation were dissolved in 95% ethanol and added to the liver cells by means of lambda pipettes. After addition of the chemicals the culture vials were returned to the incubator for twenty-four hours.

MEASUREMENT OF FLUORESCENCE INTENSITY

The cover slips were removed from the culture vials, inverted onto microscope slides and gently blotted dry with a filter paper. The cover slips were sealed to the slides with molten paraffin, washed with distilled water and examined for the presence of porphyrins with a fluorescence microscope.

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